STUDIES

FROM THE INSTITUTE OF MEDICAL CHEMISTRY UNIVERSITY SZEGED

VOL II.

(1942)

EDITED BY

A. SZENT-GYÖRGYI

(Sent to press on Dec. the 22nd 1942)

S. KARGER

BASEL, NEW YORK.

SZEGED VÁROSI NYOMDA RT. Felelős nyomdavezető : Klenóczky József

Actin.

by

F. B. Straub.

It has been shown by *Banga* and *Szent-Györgyi*[1] that myosin can be extracted from rabbit's muscle in two different forms.

Myosin A is obtained by extracting the muscle tissue with three volumes of *Weber*'s solution* for 20 minutes in the cold and centrifuged immediately thereafter. The solution of myosin A obtained in this way is viscous and threads may be prepared from it. Neither the viscosity of the solution, nor the threads prepared from it, show any significant change on adding adenyltriphosphate (ATP).

If the muscle is extracted in a similar way for 20 minutes with the same solution, but left to stand for 24 hours in the cold and centrifuged only thereafter, a turbid and very viscous solution is obtained. On addition of ATP the viscosity of such a myosin B solution is decreased to a great extent. Threads prepared from myosin B show a vigorous contraction on addition of ATP and in presence of definite amounts of salts such as KCl and $MgCl_2$.

There was another difference between the two myosin modifications. Whereas both of them would split adenyltriphosphate with the appearence of free posphate, this enzyme action was increased by Mg ions in the case of myosin B, and not increased but rather inhibited in the case of myosin A.

The problem of understanding the difference between these two modifications of myosin was taken up by studying

* This solution contains 0,6 M KCl, 0,04 M $NaHCO_3$ and 0,01 M Na_2CO_3. In previous communications from this Institute (Studies from the Institute of Medical Chemistry, Szeged, vol *1)* this solution was referred to as *„Edsall*'s solution". Since then it was brought to our attention that this solution was first used by *Weber* and *Meyer*[2].

the factors bringing about the transformation of myosin A into myosin B. These investigations led to the discovery of a new protein present in the muscle stroma. The name *actin* was given to this protein. In combination with myosin it gives the contractile protein of the muscle. As shown later in this paper, myosin B is formed if a certain amount of myosin A and actin are mixed. It follows that myosin A is what was termed by earlier investigators as myosin, myosin B on the other hand is a mixture of a definite amount of actin and myosin.

The ability of a myosin preparation to react with a decrease of viscosity on addition of ATP, was termed by *Banga* and *Szent-Györgyi* the „activity" of the respective myosin. It will be shown in this paper that apart from myosin B other mixtures of myosin and actin can be prepared, which show varying degrees of activity. Myosin B is only one of the possible combinations, its significance being only that if the muscle is extracted in the way described above, myosin B vill be invariably extracted. But muscle does not contain myosin B, instead it contains an actin-myosin complex with a higher activity than myosin B. We therefore think it advisable to modify the nomenclature put forward by *Szent-Györgyi*[3] in such a way that *actomyosin* is generally a mixture or compound of actin and myosin, there being many possible actomyosins. One of them is myosin B.

Methods.

The viscosimeters used in this work had the following measurements: capillary diameter 0,060 cm, length of capillary 210 mm, diameter of the cylindrical reservoir tube 1,65 cm, amount of outflowing fluid 1,2—1,7 ml. The viscosimeters have been placed in an icebath, which was vigorously stirred. The time of outflow of the solution was referred to the time of outflow of the solvent. No correction was taken for the change of specific weight by the presence of the proteins, as the protein content was maximally 3 mg/ml. All measurements have been performed using a buffered KCl solution of pH 7 as solvent. (Its composition see in the following paper of *Balenovič* and *Straub.)* 4 ml of myosin solution were placed in the viscosimeter. ATP was added in the form of its K salt, 0,1 ml of a 1,4% solution were added to 4 ml solution.

Determination of actin in solution.

Any specific property of actin which is in any way proportional to its quantity, may be utilized for its quantitative determination. The combination of actin with myosin to form an actomyosin is such a specific property. The activity of the resulting actomyosin is the higher, the more actin is added to the myosin. It remains to define the measure of the activity of actomyosin. This is complicated by the fact that the decrease of viscosity on addition of ATP depends not only on the actin content but also on the viscosity of the actomyosin. This is cleary brought out in Fig. 1. It shows the decrease of the specific viscosity ($\Delta\eta_{sp}$) on addition of ATP as the function of the specific viscosity in presence of ATP (η_{ATP}). The curve is valid for myosin B and it was constructed from the data of *Balenovič* and *Straub* (Fig. 1. of the following paper).

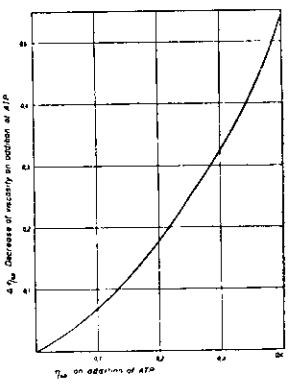


Fig. 1.

The activity of an unknown myosin solution is defined as the relation of its $\Delta\eta_{sp}$ to the $\Delta\eta_{sp}$ of a myosin B solution having the same η_{ATP} value.

As example let us take a myosin solution, which has the specific viscosity $(\eta) = 0{,}625$, in presence of ATP $(\eta_{ATP}) = 0{,}35$. Fig. 1. shows that in case of myosin B to this value of η_{ATP} corresponds a $\Delta\eta_{sp}$ of 0,41. After the above definition the activity is

$$\frac{0{,}625 - 0{,}35}{0{,}41} \cdot 100 = 67\,\%$$

To a myosin A solution,* which contains 6 mg of myosin, and has no activity (no decrease of viscosity on addition of ATP) different amounts of an actin solution are added, the solution is made up to 6 ml so as to have 0,6 M KCl concentration and pH 7, and the activity of the resulting mixture is

* All experiments described in this paper have been performed using crude myosin A solution, obtained by extracting the muscle tissue with the *Weber* solution.

determined in every case. The results are plotted against the amount of actin as in Fig. 2. From the curve it is easy to obtain the amount of actin which is necessary to transform the 6 mg of myosin present in the experiment into myosin B (100% activity). From the purest actin so far obtained 1 mg protein is needed to transform 6 mg of myosin into a 100% active myosin. The actin content of an unknown actin solution can be therefore evaluated if we determine the amount of the solution necessary to activate 6 mg myosin from 0 to 100%. In this way the element of arbitrariness introduced by the arbitrary measure of activity, is again eliminated.

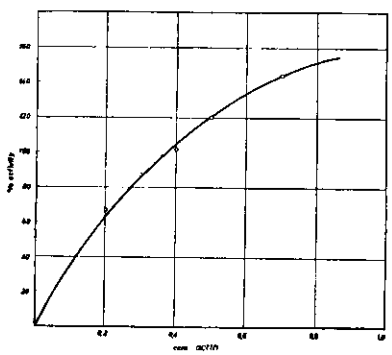


Fig. 2.

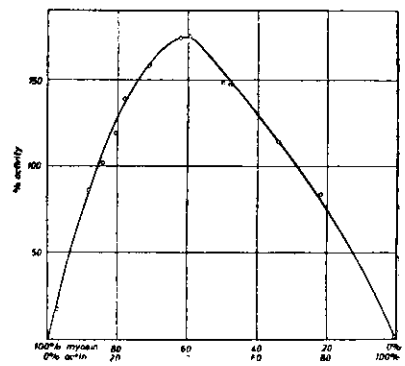


Fig. 3.

There are good reasons to believe that the actin preparations, of which 1 mg activates 6 mg myosin to a 100% active actomyosin, represent the pure actin. This means that myosin B is a compound of 6 mg myosin and 1 mg actin.

That myosin B is a compound of myosin and actin, is supported by the following experiment, in which actin was prepared from myosin B. In the usual way a myosin B was obtained, dissolved in *Weber*'s solution. It was diluted with 5 volumes of distilled water and centrifuged. The precipitate was then treated first with 4 and then with one volume of acetone and left to dry at room temperature. By extracting the dried myosin with distilled water a viscous solution of actin is obtained, which does not contain any myosin. $^1/_4$-$^1/_3$ of the estimated actin contents of myosin B can be extracted in this way.

It is seen from Fig. 2. that on addition of more actin than necessary to bring about 100% activation, actomyosins may be

obtained with more than 100% activity. Fig. 3. shows the extension of these studies. It is seen that addition of more and more actin, i. e. increasing the ratio actin: myosin, results first in an increase of activity and later in a decrease of it. Finally, actin alone like myosin alone does not show any change on addition of ATP.

The existence of actomyosins more active than myosin B is rather important. It will be described in the following paper that muscle contains an actomyosin with 170% activity. It is seen from Fig. 3. that this is the maximal activity to be achieved at pH 7.

Preparation of actin.

Rabbit's muscle from the legs and of the back are minced in a cooled Latapie mincer. To every 100 g are added 300 ml of *Weber*'s solution and stirred mechanically for 20 minutes at 0°. The mixture is centrifuged and the supernatant solution discarded. The residue is left to stand in the cold room for one or two days. It is then stirred up with 5 times its volume of distilled water and centrifuged again. The solution is again discarded and the residue mixed with 4 volumes of acetone. After 10 minutes the acetone is sucked off and replaced by a fresh lot of 1 volume of acetone. After 10 minutes this is sucked off again and the residue is spread over a filter paper and left to dry at room temperature over night.

The acetone dried muscle is extracted with 20 volumes of distilled water. The extraction can be carried out in either of the two following ways:

a) The dried muscle is mixed with the distilled water and then left to stand in itself without stirring for 4 hours at room temperature.

b) The dried muscle is ground with the distilled water in a mechanical mortar. In about $^1/_2$—1 hour, depending on the amount to be extracted, the mixture will become a rather rigid mass of foam. Further grinding is then inadvisable as the actin is denatured when present in the foam. During the subsequent centrifugation the foam is reduced to a viscous liquid.

After the extraction the undissolved muscle particles are centrifuged off; the resulting solution contains the actin. There

is no difference in the purity of the actin obtained by either of these procedures. But whereas by the thorough grinding nearly all of the actin is extracted, by procedure a) only about $^1/_6$-$^1/_4$ of the estimated actin of the muscle can be obtained in solution. On the other hand, actin obtained by a) is a clear solution, that obtained by b) is a stable milky suspension. Usually these solutions contain 3—4 mg protein per ml and the purity is such that 1,5—2 mg activate 6 mg myosin to 100%. From these solutions of the actin further purification can be achieved by precipitating it isoelectrically or by Ca ions.

The actin solution, obtained by procedure a) is diluted 8 times by distilled water and then an acetate buffer of pH 4,8 is added. The buffer concentration will be 0,01 M. The precipitate is centrifuged off at room temperature and dissolved by the addition of bicarbonate to neutral reaction.

Actin can be precipitated by adding $CaCl_2$ solution to the actin solution. The amount of Ca necessary to precipitate the actin however depends largely on the concentration of univalent ions already present. Thus about 0,02 M $CaCl_2$ is needed to precipitate the actin of the first extract (which still contains some of the KCl of the *Weber*'s solution), but 0,002 M or less is sufficient to give complete precipitation from isoelectrically purified actin solutions, which are poor in salt.

The Ca-precipitate of actin is inactive. Its activity will however return if KCl is added to the solution. At the same time it is observed that the Ca precipitate will form a stable suspension if KCl is added to it. To ensure this dissolution and reactivation, 40—50 times as much K^+ ion should be added as there was Ca^{++} ion present.

Obviously, the facts recorded here, can be described as a Ca : K antagonism. As the action of Ca is very pronounced and is observed with small, physiological concentrations, moreover the ratio Ca : K, where the effect of Ca is abolished is close to the physiological values, further, considering that actin is a part of the contractile element of the muscle, we may suppose that these observations are related to the Ca : K antagonism in muscle physiology. Further work is planned along this line.

It should be mentioned here that not only Ca^{++}, but also Mg^{++}, Mn^{++} or Sr^{++} ions can precipitate the actin in the

same way. On the other hand the effect of Ca^{++} can be overcome by Na^{+} ions just as well as by K^{+}.

It appears that the colloidal state of actin does not materially influence its activity. It was mentioned before that a clear solution obtained by extraction a) activates myosin to the same extent as the suspension obtained by extraction b), if both have an equal protein content. For this reason we prefer to prepare actin by the first method and such preparations have been used throughout this study. If the clear actin solution is treated with acid to precipitate it at pH 5—6, when dissolved at ph 7 the actin does not form a clear solution any more, but is more or less cloudy. When precipitated with Ca and redissolved with KCl, it is always a suspension.

The isoelectric precipitation results in removing 10—15% of the protein and the activity of the preparation is raised sometimes by more than 50%. Whether this is due to the removal of inhibitors or some other factor could not be decided. An actin, purified through the Ca-salt, has the maximal activity and all of its protein is precipitated at pH 6 or by new addition of Ca.

As other purification procedures, like precipitation with alcohol or salting out with KCl did not lead to further purification and because all of the protein was precipitated by minimal Ca concentrations, we think that such preparations are homogeneous and do not contain any significant amount of impurities.

Actin is easily destroyed by heat over 50°. It is stable in a narrow pH zone between pH 7—7,5 but is rapidly destroyed even at 0° by more acid and alkaline reactions. It is precipitated from the solution at pH 6. Dialysis at pH 7 does not diminish its activity. From the solution it can be precipitated by cold alcohol but it is completely destroyed if treated with cold acetone. (This is the more interesting as it is very resistent to acetone when still in the muscle tissue.) If precipitated without loss of activity, the precipitate is voluminous. After centrifuging for 20 minutes at 3000 r. p. m. it still contains more than 99,5% of water.

Thixotropy. The most remarkable property of actin solutions is their very strong thixotropy. A dilute solution of neutral actin containing as little as 3—5 mg protein per ml sets

to a gel if left alone at 0° for a few hours. A slight shaking however breaks up the gel immediately. As common with thixotropic gels, the viscosity of actin solutions cannot be determined with accuracy. So much can be said only that it is roughly the same as that of myosin. The time of outflow is determined largely by the shearing forces present in the capillary viscosimeter. For this reason successive determinations of the time of outflow show strongly decreasing values. If the solution is left to stand for a while before the next determination, again a higher value of viscosity will be found. A quantitative study of these phenomena is planned in a system, in which the pressure can be taken into consideration.

Strictly speaking it would be better to use the term resistance (Strömungswiederstand) instead of viscosity. The resistance of a solution results from the true viscosity, which can be measured at high shearing forces and from the elasticity of the solution. That indeed elasticity plays an important part is shown by the fact that the viscosity increases if the time of outflow is longer, i. e. the shearing forces are smaller. The resistance of actin solutions decreases assymptotically with higher shearing forces. It is interesting to point out two parallelisms between actin and thixotropic inorganic sols. One is the permanent double refraction to be dealt with later. The other is the great influence, which inorganic salts exercise on the resistance (apparent viscosity) of actin solutions. It is known from the experiment of *Freundlich*[4] and the theoretical treatment by *Szegvári*[5] that the elasticity but not the viscosity is influenced by the salt concentration.

Not only the viscosity of actin, but also the viscosity of actomyosin is influenced by the strong thixotropy of actin. Whereas myosin A solutions show no variation in viscosity during successive determinations, myosin B shows already such an effect, a second run in the viscosimeter gives always lower value than the first one. This results in some ambiguity in the determination of viscosities. The more active an actomyosin is, the less reliable is the determination of viscosity. Therefore we have accepted a certain routine, which would give comparable results. The actin solution was kept at 0° for at least 1 hour prior to use, then it was gently shaken up and the desired amount mixed with the myosin, the mixture imme-

diately put into the icebath and its viscosity determined 5 minutes later. Immediately thereafter two more readings were taken. After the addition of the ATP, two more determinations were made. For the calculation of the activity the first readings were always used. The others served only to see the extent of the thixotropic effect. The solutions are to some extent thixotropic even after the addition of ATP.

Whereas the difficulties caused by the thixotropic effect make the viscosity values uncertain, the determination of activity by this routine procedure can be carried out with satisfying reproducibility, except for actomyosins containing more actin than myosin.

A thixotropic effect with salt free myosin has been observed already by *Muralt* and *Edsall*.[6] It is clear that these authors dealt with a myosin A preparation, that is an actomyosin of a few per cent activity. This is shown by the very small dependence of the viscosity of their solutions on the shear rate. *Freundlich*[7] has suggested that the interior of the muscle cell is a thixotropic gel, as evidenced by the observation of *Kühne*.[8] Knowing that the myosin in the muscle fibril is not the myosin studied by *Muralt* and *Edsall*, but an actomyosin of about 170% activity, which is a rather strongly thixotropic substance, this assumption is now experimentally supported.

Thixotropic behaviour is an expression of certain properties of actomyosin, which might be of considerable significance in understanding the architecture and the mechanical properties of muscle.

If we assume that the most active actin, described in this paper, is the pure actin, it was calculated (see *Balenović* and *Straub*) that 1 gramm of muscle contains 25—30 mg actin. It is impossible to make such a concentrated solution of actin, which requires a very close packing. Actin reveals by its thixotropy and permanent double refraction (see later) very strong intermolecular forces. At the close packing present in muscle, these intermolecular forces must be very strong and must play an important rôle in determining the mechanical properties of muscle.

High viscosity and thixotropy are two phenomena each pointing to the fact that actin has elongated, rod-shaped molecules of very great assymetry. Double refraction of flow is

another typical characteristic of actin solutions, bearing evidence to the same point. The sign of double refraction is identical with that of myosin. Myosin already has a strong double refraction of flow, which appears at very low shearing forces. But its double refraction is far weaker than that of actin solutions. A slight movement of the actin solution gives rise to the appearence of doubly refracting patches, which persist long after the fluid had ceased to move. If there is so much actin in solution that it turns into a thixotropic gel, the double refraction is permanent. It is interesting to remember that according to *Freundlich* and *Schalek*,[4] thixotropy is observed among those inorganic sols (like aged V_2O_5 sols) which show a permanent double refraction.

Of the physicochemical properties touched upon in this paper, viscosity and double refraction of flow show a parallelism with the actin content of the solution.

The process of activation.

If actin is mixed with myosin in solution, an actomyosin will be formed instantaneously. When in contact with muscle, myosin will be activated at 0° only in the course of many hours. It has been pointed out by *Banga* and *Szent-Györgyi*[1] that there is no activation at all, until the ATP of the muscle is split. That the disappearence of ATP is not the only factor involved in determining the rate of activation, is demonstrated by the following experiment. Rabbit's muscle is extracted at 0° several times in succession with *Weber*'s salt solution. By 3—4 extractions, practically all of the myosin is extracted. On adding an ATP-free myosin solution to the residue, which is by now likewise free of ATP, activation of myosin takes again nearly 24 hours at 0°. The rate of activation can be, however, strongly increased by grinding the muscle residue with sand, prior to the incubation with myosin. In this case activation will be complete within several minutes. From these experiments it follows that the rate determining process of the activation in muscle is the extraction of the actin from the muscle residue.

In absence of myosin, the *Weber* solution will extract no actin from the muscle residue. The presence of myosin is

therefore essential for the extraction of actin. In absence of ATP, as shown by *Banga* and *Szent-Györgyi*,[7] neither myosin, nor actin are extracted.

To account for these phenomena, we must assume that the actomyosin present in the muscle, dissociates into its components in presence of ATP and the salts of the *Weber* solution. The myosin is dissolved but the actin is retained by the strong intermolecular forces. After the ATP has disappeared, the myosin already in solution forms a compound again with the actin. By forming this compound, the forces binding actin in its place are overcome and the dissolved myosin dissolves the actin.

The only other way, by which I succeeded to liberate actin is the acetone treatment described above. But even acetone is capable of breaking up the structure only if the latter has been loosened up by a prolonged treatment with the alkaline salt solution.

Actin is not extracted by the usual salt solutions which have been used to study the distribution of muscle proteins. Therefore it is clear that only a fraction of it goes into solution (this gives the few % activity of myosin A), whereas the greater bulk of it remains in the so called muscle stroma. This insoluble protein fraction has been estimated as 15—20% of the muscle proteins. We find (see *Balenovič* and *Straub*) that actin represents about 12—15% of the muscle protein. Making an allowance for some connective tissue and the nucleoproteids of the nuclei there is not much protein left unaccounted. We therefore find that there is no other protein left in the muscle stroma, to which the rôle of a structure-protein could be assigned. The possibility that actin is the main structure-protein, receives strong support by its properties, discussed above.

The formation of the myosin-ATP complex.

It has been shown by *Mommaerts*[9] that about one gramm molecule of ATP are needed to give a maximal effect with 100,000 gramm of myosin. From the experiments of the author[10] on myosin A at acid reaction, it became obvious that ATP is bound to the myosin part of the actomyosin complex. This

is supported also by the fact that myosin is the carrier of the adenyltriphosphatase activity.

On a 100% active actomyosin, prepared from myosin A and actin, I have reinvestigated this problem. As *Mommaerts'* experiments were performed at the alkaline reaction of the *Weber* solution, it was important to know the conditions at physiological pH. The viscosities have been determined at pH 7,0 in a solution containing 0,6 M K ions and veronal acetate buffer. The decrease of viscosity on addition of ATP was

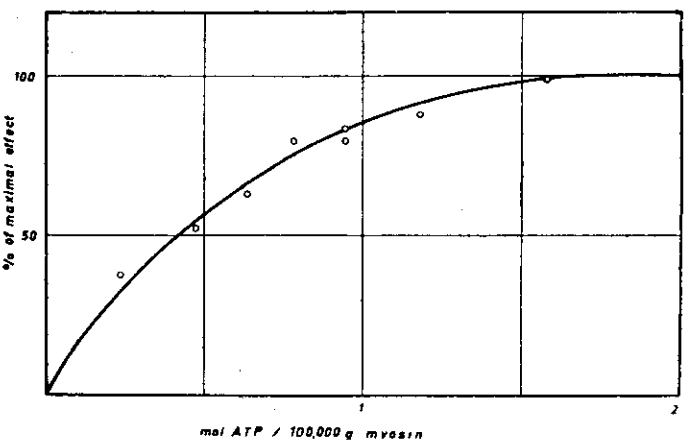


Fig. 4.

measured and the results are expressed as % maximal effect and plotted against the amount of ATP in Fig. 4. 0,1 ml ATP solution was added in each case to the 6 mg of actomyosin present in 4 ml salt solution. From four successive determinations after the addition of ATP, the viscosity of the solution at the moment of adding the ATP was determined by linear extrapolation. It is evident, from the nature of the curve that the bond between myosin and ATP is very strong, less than 2 g molecules of ATP being needed for 100,000 g of myosin B to give a maximal effect. There is some dissociation of the myosin-ATP complex, but the results are essentially in agreement with the assumption that 1 g molecule of ATP reacts with 100,000 weight of myosin.

It is quite convenient to work with 6 mg of myosin in the 4 ml of the solution placed in the viscosimeter. In this case about 0,015 mg ATP would give a 50% effect. In our experi-

ments such an amount of ATP caused the viscosity of the myosin to decrease from 1,83 to 1,61. Such a change in viscosity can be determined with satisfactory precision. Considering the absolute specificity of ATP in decreasing the viscosity of myosin, these facts may be utilized for determining very small amounts of ATP.

Summary.

A method for extracting a new protein, actin, from muscle tissue is described. Actin forms, together with myosin, actomyosin, the contractile protein of muscle. The most conspicuous properties of actin are its high viscosity, thixotropy and strong double refraction, all proving a great molecular assymetry.

The antagonistic effect of Ca and K ions on actin is described.

References:

1. *I. Banga* and *A. Szent-Györgyi,* Studies from the Institute of Medical Chemistry, Szeged, *1,* 5 (1942).

2. *H. H. Weber* and *K. Meyer,* Biochem. Z. *266,* 137 (1933).

3. *A. Szent-Györgyi,* Studies from the Institute of Medical Chemistry, Szeged, *1,* 67 (1942).

4. *H. Freundlich* and *E. Schalek,* Z. physik. Chem., *108,* 153 (1924).

5. *A. Szegvári,* Z .physik. Chem. *108,* 175 (1924).

6. *A. L. v. Muralt* and *J. T. Edsall,* J. biol. Chem., *89,* 315 (1930).

7. *H. Freundlich,* Protoplasma, *2,* 278 (1927).

8. *A. Kühne,* Virchow Arch. path. Anat., *26, 222* (1863).

9. *W. F. H. M. Mommaerts,* Studies from the Institute of Medical Chemistry, Szeged, *1,* 37 (1942).

10. *F. B. Straub,* ibid., *1,* 43 (1942).

Über das Aktomyosin des Kaninchenmuskels.

von

K. Balenovic und F. B. Straub.

Aktomyosin ist ein Komplex von Myosin und Aktin. BANGA und SZENT-GYÖRGYI[1] haben einen solchen Komplex aus Kaninchenmuskulatur erhalten, den sie Myosin B benannten. Es wurde von STRAUB[2] bewiesen, dass dieser Komplex aus Myosin und Aktin besteht. Nach den Angaben der vorangehenden Arbeit können durch Mischen von Myosin mit verschiedenen Mengen von Aktin Aktomyosine erhalten werden, die durch verschieden grosse Viskositätsänderungen auf Zusatz von Adenyltriphosphat (ATP) reagieren. Die Grösse dieser Viskositätserniedrigung wurde als Mass der „Aktivität" eines Aktomyosins definiert. Die Aktivität von Myosin B, die bei den verschiedenen Präparaten konstant ist, wurde willkürlich 100% Aktivität genannt. STRAUB beobachtete, dass auch Aktomyosine von mehr als 100% Aktivität entstehen können, wenn man mehr Aktin dem Myosin zusetzt.

Wir haben gefunden, dass in einigen Fällen bei der Darstellung des Aktins aus dem Muskel soviel Aktin ausgelöst werden kann, dass dieses das im selben Muskel vorhandenen Myosin mehr als 100% zu aktivieren vermag. Dadurch müssten wir die Möglichkeit in Betracht ziehen, dass das im Muskel vorhandene Aktomyosin, also das eigentliche Kontraktionsprotein, aktiver ist als Myosin B. In dieser Arbeit wird gezeigt, dass das Aktomyosin des Kaninchenmuskels wahrscheinlich 170% aktiv ist und enthält 1 mg Aktin pro 3 mg Myosin. Ist dem aber so, dann müsste eine weitere Frage beantwortet werden: warum erhält man bei der Extraktion des Muskels mit der WEBER-Lösung ein Aktomyosin (Myosin B) das im Muskel nicht vorkommt?

Da Aktin in saurer oder alkalischer Lösung rasch zer-

stört wird, schien es zweckmässig die Viskositätsmessungen nicht in WEBER-Lösung, sondern bei pH 7 auszuführen. Deshalb haben wir die Viskositätsbestimmungen von BANGA und SZENT-GYÖRGYI,[1] die in WEBER-Lösung ausgeführt wurden, wiederholt. Dieselben Kapillarviscosimeter, die in der vorangehenden Arbeit beschrieben wurden, dienten zu diesen Messungen. Myosin wurde in einer 0,6 m KCl Lösung gelöst, die aber durch Veronal-Azetat auf pH 7,0 gepuffert war. Diese Lösung wurde folgenderweise bereitet: zu 200 ccm der Veronal-Azetat Mischung nach MICHAELIS (die aber Kalium an Stelle von Natrium enthält) wurden 24 ccm n-HCl zugesetzt, dann 271 ccm einer 2 m KCl Lösung und auf 1 Liter aufgefüllt. Die K Ionenkonzentration war dann 0,6 m.

Myosin A und B wurden folgenderweise hergestellt: Kaninchenmuskulatur wurde durch einer eisgekühlten Latapie-Mühle gemahlen und bei 0° mit 3 Volumen WEBER-Lösung unter mechanischen Rühren 20 Minuten lang extrahiert. Zentrifugiert man die Mischung nach dieser Zeit, so erhält man eine Myosin A Lösung. Wird die Mischung nach dem Extrahieren 24 Stunden lang bei 0° stehen gelassen, mit dem gleichen Volumen WEBER-Lösung verdünnt und dann zentrifugiert, so bekommt man eine Lösung von Myosin B. Solche Lösungen wurden durch vorsichtige Zugabe von einer 2 m Azetatpuffer-Lösung auf pH 7 neutralisiert und mit der oben beschriebenen gepufferten KCl Lösung von pH 7 entsprechend verdünnt.

Der Myosingehalt der Myosin B Lösungen wurde in der von BANGA und SZENT-GYÖRGYI angegebenen Weise bestimmt. Bei der Bestimmung des Myosingehaltes von Myosin A Lösungen haben wir aber beobachtet, dass die Trockengewichtsbestimmung in der von BANGA und SZENT-GYÖRGYI angegebenen Weise schwankende Ergebnisse gibt. Bei der Neutralisierung der verdünnten Myosinlösung kann nämlich das pH schwer auf pH 7 eingestellt werden. Wir haben versucht, diese Trockengewichtsbestimmung zu standardisieren. Gibt man zu je 1 ccm einer Myosin A Lösung (in der WEBER-Lösung gelöst) 5 ccm 0,1 m Azetatpufferlösungen von verschiedenen pH, so erhält man Niederschläge von Myosin, deren Menge mit dem pH variiert und einen maximalen Wert bei Zugabe einer pH 4,8 Azetatpufferlösung erreicht. Das pH der Lösung ist

wegen der Alkalinität der Myosinlösung etwas höher, 5,2. Tabelle 1 zeigt die Ergebnisse dieser Versuche. Myosin I ist ein gereinigtes Myosin, Myosin II ein ungereinigtes Myosin, beide in WEBER-Lösung gelöst.

Tabelle I.

pH der zugegebenen Puffers	Trockengewicht mg/ccm	
	Myosin I.	Myosin II.
3,6	0,7	1,3
4,0	1,1	3.7
4,4	8,0	21,9
4,8	9,1	22,5
5,2	8,4	19,0
5,6	8,6	17,4

Wir haben deshalb die Myosin A Trockengewichte derart bestimmt, dass wir immer 1 ccm des sich in WEBER-Lösung befindenden Myosins mit 0,5 ccm 0,1 m Azetatpuffer von pH 4,8 versetzten. Der Niederschlag wurde auf der Zentrifuge zweimal mit Wasser gewaschen, bei 105° getrocknet und gewogen.

Fig. 1. zeigt die Ergebnisse unserer Viskositätsmessungen. Im Wesentlichen erhält man dasselbe Bild, wie in der Arbeit von BÁNGA und SZENT-GYÖRGYI. Es gibt nur den Unterschied, dass wir die Viskosität von Myosin A und B in Gegenwart von ATP gleich gefunden haben.

Versuchsvolum in dem Viskosimeter war 4,0 ccm, dazu wurden 0,1 ccm einer 1,4%-ger ATP-Lösung gegeben. Temperatur: 0°.

In die Figur wurde die Viskosität von Myosin A ohne Zugabe von ATP nicht eingezeichnet. Dieser Wert variiert. Wir hatten Präparate die keine Aktivität besassen (keine Viskositätsänderung auf Zugabe von ATP) und andere die 20% aktiv waren. Meistens findet man aber eine Aktivität von 15% bei Myosin A. Aus verschiedenen Gründen nehmen wir an, dass diese Aktivität durch ein wenig Aktin hervorgerufen wurde, das als Verunreinigung in Myosin A Präparaten vorkommt. Reines Myosin hat demgemäss keine Aktivität.

Den Zusammenhang zwischen Viskosität von Myosin B in Gegenwart von ATP und die durch ATP hervorgerufene Viskositätserniedrigung stellt Fig. 1. der voranstehenden Ar-

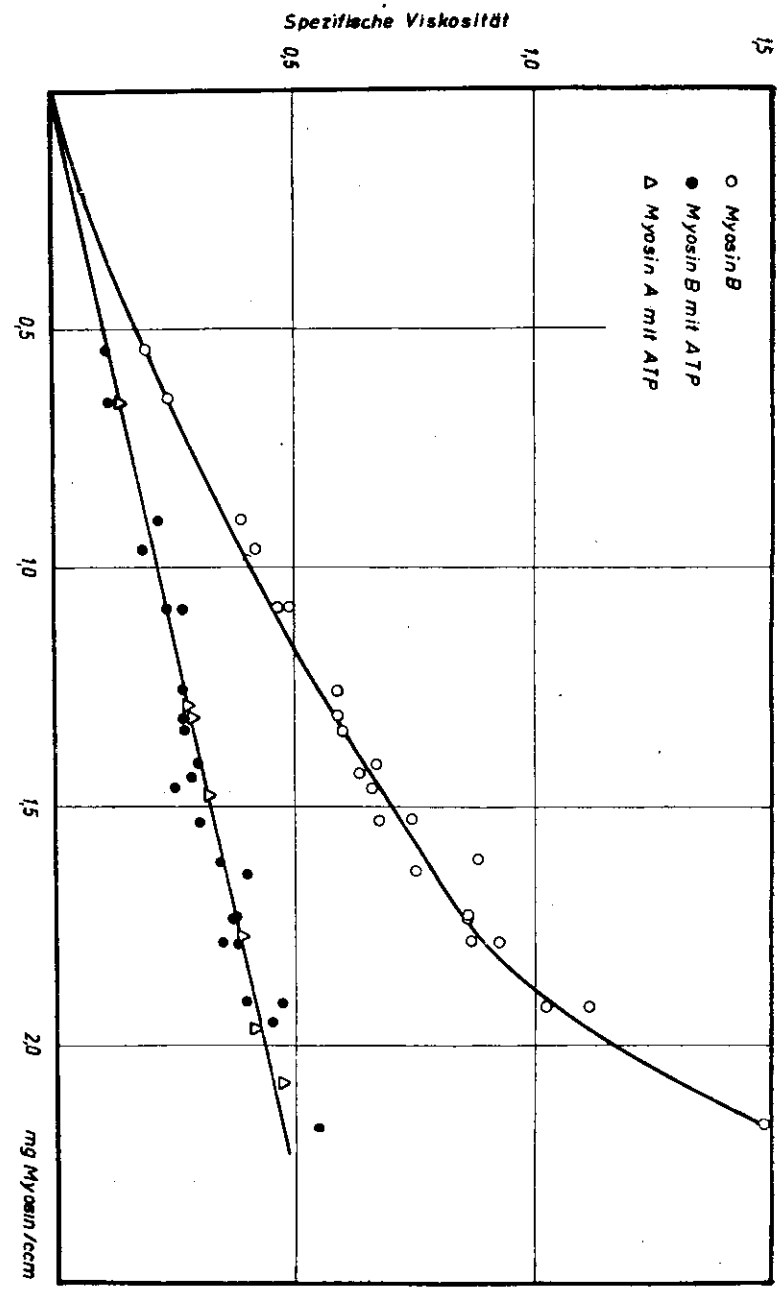

Spezifische Viskosität
0,5
1,0
1,5
○ Myosin B
● Myosin B mit ATP
△ Myosin A mit ATP
0,5
1,0
1,5
2,0
mg Myosin/ccm

Fig. 1.

heit dar. Auf diesem Grunde wurde die Methode der Auswertung der Aktivität eines unbekannten Aktomyosins ausgearbeitet. (Näheres s. bei STRAUB[2].)

Bestimmt man die Aktivität eines Myosin B Präparates, das nach der oben beschriebenen Methode dargestellt wurde, so findet man innerhalb der Fehlengrenzen der Methode immer eine 100% Aktivität. Wurde das Myosin mit dem Muskel nicht 24 sondern 48 Stunden lang stehen gelassen, dann verdünnt und zentrifugiert, so findet man meistens eine 100%, manchmal aber eine kleinere Aktivität. Das Myosin, das nach drei Tagen vom Muskel abgetrennt wird, ist schon immer weniger als 100% aktiv, z. B. in einem Falle nur 60%. Abgesehen von dieser Aenderung der Aktivität nach längerem Stehen, ist es klar, dass in erster Linie nicht eine Zeitfaktor in der Entstehung von Myosin B eine Rolle spielt. Myosin B, wenigstens nach der beschriebenen Methode dargestellt, ist ein wohl definiertes Produkt und reagiert bei Zugabe von ATP mit einer konstanten Viskositätserniedrigung.

Bestimmung des Aktins im Kaninchenmuskel. Bei der Darstellung des Aktins wird die Muskelsubstanz zuerst bei alkalischer Reaktion gehalten und dann mit Azeton behandelt. Beide Operationen können zu unkontrollierbaren Verlusten führen. Deshalb kann man aus Ausbeute bei der Isolierung auf die Menge des Aktins keinen Rückschluss ziehen. Mit der Methode von STRAUB wird durchschnittlich soviel Aktin aus dem Muskel isoliert, wie zur 100% Aktivierung von 30—60% des im Muskel vorhandenen Myosins nötig wäre.

Folgendes Verfahren ist aber für die Bestimmung des im Muskel gebundenen Aktins geeignet: man bestimmt die Menge an Myosin, die durch 1 g Muskel 100% aktiviert werden kann.

Kaninchenmuskel wurde wie üblich mit 3 Volumen WEBER-Lösung 20 Minuten lang extrahiert. Die Mischung wurde zentrifugiert und das Myosin A vom Muskelrückstand abgetrennt. Aus 150 g Muskel (Frischgewicht) erhielten wir somit 340 ccm von Myosin A Lösung und 240 g Rückstand. Die Lösung enthielt 22,5 mg/ccm Myosin.

Nun wurde die Hälfte des Rückstandes (der das Aktin enthält) mit der gesamten Menge Myosin zusammengebracht,

24 Stunden lang bei 0° stehen gelassen, dann mit WEBER Lösung verdünnt und zentrifugiert. Die Aktivität der überstehenden Myosinlösung wurde aus dem ATP Effekt viskosimetrisch bestimmt. Solche Versuche wurden auch derart eingestellt, dass 20, 25, 30, 33, 40, 100 und 200% des Rückstandes mit der gesamten Myosinlösung gemischt wurden. In jedem Falle wurde die Aktivität nach 24 Stunden bestimmt. Die Ergebnisse solcher Experimenten mit verschiedenen Muskelpräparaten sind in der Fig. 2. zusammengestellt. Man sieht, dass ungefähr $^1/_3$ des Muskelrückstandes genügt, um die ganze Myosinlösung 100% zu aktivieren. Ist mehr Rückstand, also mehr Aktin vorhan-

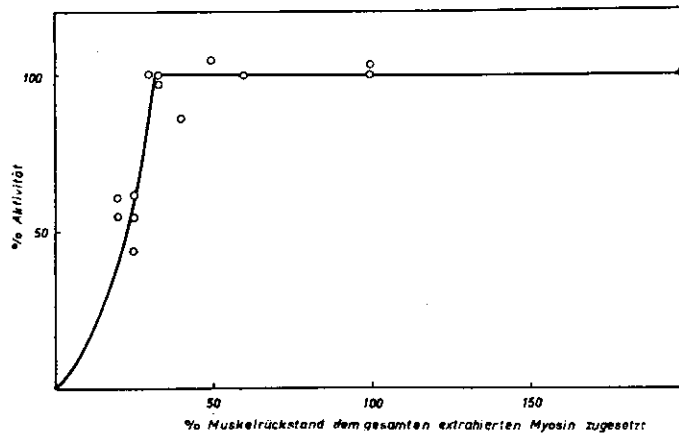


Fig. 2.

den, so tritt keine weitere Aktivierung ein. Dies ist im Gegensatz zur Aktivierung bei pH 7 mit gelöstem Aktin wo die Aktivierung bei weiterem Zusatz von Aktin bis 170% weitergeht.

Bei der zahlenmässigen Auswertung dieser Ergebnisse darf man nicht ausser Achtung lassen, dass ein Teil des Myosins durch die einfache Extraktion mit 3 Volumen WEBER-Lösung im Rückstand zurückbleibt. Die Schwankungen in der Menge des extrahierten Myosins sind für die Streuung der Punkte in Fig. 2. verantwortlich. Ist M die Menge des Myosins pro g Muskel und werden davon M' pro g extrahiert, so bleibt im Rückstand $M—M'$ pro g. Bekommt man mit 33% des Rückstandes noch eben eine 100% aktive Myosinlösung, so bedeutet das, dass das Aktin von 0,33 g Muskel 0,33 $(M—M') + M'$ Myosin 100% aktivierte. In einem unserer Versuche aktivier-

ten 33% des Rückstandes noch eben zu 100%. M' war 51 mg/g, M ist bekanntlich 80 mg/g. Das Aktin des 0,33 g Muskels aktivierte also

$$0{,}33\ (80—51) + 51 = 60\ \text{mg Myosin},$$

1 g Muskel dreimal soviel, also 180 mg. Nach den Angaben von STRAUB braucht man 1 mg Aktin um 6 mg Myosin 100% zu aktiveren. Daraus folgt, dass im Muskel 180/6 also 30 mg Aktin pro g (Frischgewicht) gibt. In ähnlichen Versuchen fanden wir den Wert 26—30 mg/g Muskel. Zu einer 100% Aktivierung, also zur Entstehung von Myosin B wäre die Hälfte dieser Menge nötig. Nach den Angaben von STRAUB[2] bekommt man ein 170% aktives Myosin, wenn man Myosin und Aktin in der Proportion, wie sie im Kaninchenmuskel vorkommen, bei pH 7 zusammensetzt. Das ist eben der maximale Wert der Aktivität, der bei pH 7 erreicht werden kann. Demgemäss können wir sagen, dass sich im Muskel nicht ein 100% aktives Aktomyosin (Myosin B) sondern ein maximal aktives Aktomyosin mit 170% Aktivität befindet.*

Es frägt sich nun, warum man aus dem Muskel durch Extraktion mit WEBER-Lösung das 100% aktive Myosin B an Stelle des natürlichen 170% aktiven Aktomyosins erhält. Aus den in dieser Arbeit mitgeteilten Versuchen geht hervor, dass wenn auch das in WEBER-Lösung gelöste Myosin mehrere Tage lang mit dem Muskel in Berührung steht, keine Aktivierung über 100% stattfindet. Folgender Versuch zeigt, dass dabei freies Aktin im Muskel zurückgeblieben ist. Es wurde wie üblich Myosin B bereitet und vom Muskelrückstand abgetrennt. Der so erhaltene Muskelrückstand wurde nun mit soviel Myosin A versetzt, wie ursprünglich im Muskel vorhanden war. In 24 Stunden wurde auch dieses Myosin zu 100% aktiviert.

Myosin B bekommt man demgemäss darum, weil das Myosin unter den Bedingungen der WEBER'schen Lösung nur soviel Aktin aus dem Muskel extrahieren kann, das eben zur 100% Aktivierung nötig ist. Wie in der vorangehenden Arbeit erwähnt, ist Aktin im Muskel festgebunden, seine Auslösung geschieht in der Weise, dass es zu dem in Lösung vorhande-

* Fäden, die aus Myosin B und aus künstlich hergestelltem 160% aktivem Myosin ausgezogen wurden, kontrahierten in Gegenwart von K, Mg und ATP in annähernd gleicher Weise.

nen Myosin fester gebunden wird, als zum Muskelrückstand. Es ist leicht möglich, dass die Bindung zwischen Myosin und Aktin nur bis zu 100% Aktivität fest genug ist, um das Aktin aus seinem Verbande im Muskel loszulösen. Gibt man noch *gelöstes* Aktin zu einem 100% aktiven Aktomyosin, so entsteht ein aktiveres Aktomyosin.

Zusammenfassung.

1. Kaninchenmuskel enthält ungefähr 25—30 mg Aktin pro Gramm Frischgewicht. 12—15% des Gesamtproteins ist also Aktin. Diese Menge des Aktins vermag das anwesende Myosin maximal zu aktivieren (170% Aktivität).

2. Durch die alkalische Salzlösung von WEBER wird ein minder aktives (100%) Aktomyosin extrahiert.

Litteratur.

1. *I. Banga* und *A. Szent-Györgyi*, Studies from the Institute of Medical Chemistry, Szeged, *1*, 5 (1942).

2. *F. B. Straub, ibid., 2,* (1943).

The reversibility of the contraction of myosin threads.

by

A. Szent-Györgyi.

It has been shown in a previous paper (1) that threads of myosin B show a violent contraction if suspended in a solution containing KCl (0,05 M), $MgCl_2$ (0,001 M) and ATP (Adenyltriphosphate 0,17%). If such a contracted thread is washed out with water and supended in a solution containing 0,25 M KCl and 0,001 M $MgCl_2$ no appreciable change is observed. If ATP is added now to the solution the thread swells up within a few minutes to its original size; it becomes transparent and similar to the original uncontracted thread in all respects. If the liquid is replaced by the salt solution in which the contraction was obtained, the thread contracts again. The contraction is thus reversible and ATP is essential not only for the contraction but also for the relaxation. The thread can be brought to contraction and relaxation by the variation of the KCl concentration

Mg is essential for the contraction as well as for the relaxation. In absence of Mg the contraction is sluggish and there is no relaxation at all. Only a very slight swelling is obtained as revealed by the somewhat increased transparency. At higher KCl concentrations (in presence of ATP) the thread desintegrates without much swelling.

The Mg can be replaced by a dialysed extract of the muscle. Whether this action is due to the traces of Mg, possibly bound by the protein, or by some other substance, cannot be stated at present.

In presence of 0,1% quinine the same contraction and relaxation is obtained as in absence of this substance. As shown by I. BANGA this alcaloid greatly inhibits the phosphatase action of myosin. Both the contraction and relaxation can thus take place without the splitting of the ATP.

The same experiment were also repeated with threads prepared from 160% active myosin (see Straub's paper) prepared from myosin A and Actin. The results were similar to those obtained with myosin B with the one difference that in presence of ATP these threads dissolve at a lower KCl concentration. While a myosin B thread contracts still in a 0,15 M KCl solution (in presence of ATP and 0,001 M $MgCl_2$), is inactive in 0,2 M KCl and dissolves in 0,25 M KCl, the thread prepared from the 160% active myosin contracts in 0,05 M KCl, is inactive in 0,1 M KCl and dissolves in 0,15 KCl, is thus more sensitive to the action of this salt. As shown by STRAUB and BALENOVIČ, the myosin of the muscle is about 170% active.

The effect of ATP depends thus on the concentration of the KCl present. But ATP added in form of its K salt may increase the salt concentration sufficiently to convert its own action from a contracting to a dissolving one. This may be illustrated by the following experment: Threads, prepared from 160% myosin were suspended in 0,35 ml. of a 0,05 M KCl solution containing 0,001 M $MgCl_2$. Then 0,05 ml. of an ATP solution were added, containing 1,4% ATP, 0,05 KCl and 0,001 $MgCl_2$. A slow contraction is obtained. In a second experiment the threads were suspended in 0,3 ml of the KCl $MgCl_2$ solution and 0,1 ml of ATP was added. The threads dissolved. The concentration of the ATP thus determines its own effect, whether it will cause contraction or relaxation.

The reversibility of the contraction of myosin threads was also demonstrated by M. GERENDÁS and D. VARSÁNYI in a very neat way. (Oral communication). T. ERDÖS has shown that ATP, in presence of lower concentrations of KCl (+ Mg) causes a contraction of the threads while in presence of higher concentration it causes a dissolution. Between the two ranges of concentration there is a range in which the thread neither contracts, nor dissolves: is inactive. GERENDÁS and VARSÁNYI have shown that such an inactive thread can be brought to a rapid contraction by the simple dilution of the solvent or by the addition of Ca-permutit which binds the K.

Literature.

1. Studies from the Inst. Med. Chem. Szeged. *1* 17, 1941—42.

Über die Fibrinogen-Fibrinumwandlung.

von

K. Laki.

(Arbeit aus den Mitteln des fürstl. Esterházyschen Stipendiums.)

Vorliegende Arbeit bezweckt das Studium des zweiten Abschnittes der Blutgerinnung, der Umwandlung des Fibrinogens in Fibrin. Vor allem erwies es sich als erforderlich das Fibrinogen in möglichts reiner Form, frei von sonstigen Proteinen darzustellen und zu untersuchen welche Veränderungen durch die Einwirkung von Thrombin zustande kommen.

Isolierung des Fibrinogens.

Für die Reindarstellung des Fibrinogens verwandte ich das Oxalatplasma von Schweineblut.

Oxalatplasma. Das Gemisch von 200 ml 0,7%iger Kochsalz- und 100 ml 2%iger Na-Oxalatlösung wurde auf dem Schlachthofe unter mässigem Rühren mit Schweineblut auf 1 l aufgefüllt. Im Laboratorium wurde das Blut im Kühlraum (0° C) abzentrifugiert. Ich erhielt 500 ml Plasma.

Fällung durch Ammoniumsulphat. Dem kalten Plasma wurde 125 ml gesättigter Ammoniumsulphatlösung in kleinen Portionen zugefügt (0,20 Sättigung). Der Niederschlag wurde in der Kälte abzentrifugiert, sodann in dem Gemische von 90 ml 0,7 %iger NaCl- und 10 ml 2%iger Na-Oxalatlösung aufgelöst. Der kuchenartige Niederschlag löst sich langsam bei andauerndem Digerieren. Die erhaltene trübe Lösung wird durch Zentrifugieren geklärt.

Tricalciumphosphatadsorption. 100 ml 10 %iger Trinatriumphosphatlösung wurden 100 ml 10%iger $CaCl_2$-Lösung zugefügt. Der enstandene Niederschlag wurde abzentrifugiert, sodann in der Zentrifuge mit dest. Wasser

zweimal ausgewaschen, schliesslich in 0,7%iger NaCl-Lösung so suspendiert, dass das Gesamtvolumen 150 ml betrug. Das Fibrinogen wurde aus seiner Lösung in NaCl an 25 ml frisch bereitetem Tricalciumphosphatgel adsorbiert. Nach dem Hinzufügen des Phosphatgels zentrifugiert man die Lösung. Das das Fibrinogen mit sich führende Phosphatgel wurde mit 50 ml 0,7%iger NaCl-Lösung in der Zentrifuge gewaschen, das Fibrinogen mittels 80 ml eines Gemisches von gleichen Teilen M/5 primären und sekundärem Phosphats eluiert.

Zweite Fällung durch Ammoniumsulphat. Zwecks weiterer Reinigung wurde das Fibrinogen aus dieser Phosphatlösung mit einer $^1/_4$ Vol. gesättigter Ammoniumsulphatlösung (0,20 Sättigung) aufs Neue gefällt, sodann wurde der Niederschlag nach Abzentrifugieren in 30 ml des oben erwänten Phosphatpuffers unter andauerndem mässigem Digerieren aufgelöst. In der Regel erhielt ich eine wasserklare Lösung.

Die quantitativen Daten der Isolierung werden durch folgende Tabelle veranschaulicht.

	Fibrinogen mg in 1 ml	Fibrinogen %	Ausbeute an Fibrinogen
Oxalatplasma	3,6	10	1,800 g
Nach der ersten Ammoniumsulphatfällung	12,5	50	1,250 g
Nach der Ca-Phosphatadsorption	8,2	88,6	0,660 g
Nach der zweiten Ammoniumsulphatfällung	12,6	96,5	0,380 g

Bemerkt sei, dass von den Angaben dieser Tabelle oft auch grössere Abweichungen möglich sind, indem bereits die erste NaCl-Lösung nicht nur 50, sondern auch 65% Fibrinogen enthalten kann. Durch die Adsorption und Eluation vermag die Reinheit in der Regel 88—90% zu erreichen, wogegen dieselbe im letzten Schritte im allgemeinen 96—98% beträgt. Erwähnenswert ist, dass das Tricalciumphosphatgel unter den besprochenen Umständen aus der NaCl-Lösung 80% des Fibrinogens adsorbiert, wobei der Reinheitsgrad auf der Oberfläche des Adsorbens 80% ist.

Kristallisierung. Giesst man 10 ml der im letzten Schritte gewonnenen Lösung in die 10fache Menge dest. Wassers von 38—40° C, dann scheidet sich das Fibrinogen während des Abkühlens im Eisschranke in der Form eines schneeweissen flockigen Niederschlags ab, der unter dem Mikroskope aus

nadelförmigen Kristallen besteht.[1] Nach dem Abzentrifugieren löst sich der Niederschlag bei gelindem Erwärmen (38° C) in 8 ml Phosphatpuffer obiger Zusammensetzung langsam auf. Beim Impfen mit Thrombin gerinnt die Lösung. 1 ml dieser Lösung enthält 7,4 mg Fibrinogen und 7,4 mg Gesamteiweiss, d. h. das in der Lösung befindliche Fibrinogen hat sich vollständig in Fibrin umgewandelt. Diese Lösung ist auf die vorhergehende Weise aufs Neue kristallisierbar, die neuen Kristalle sind indessen schwerer löslich, was darauf hinweist, dass das Fibrinogen sich einigermassen verändert hat.

Fibrinogenbestimmung.

Wir bestimmten das Fibrinogen durch Wägen des auf Einwirkung von Thrombin entstandenen Fibrins. Zu diesem Zwecke wird die zu untersuchende Lösung in ein abgewogenes Zentrifugenröhrchen (65 × 11 mm) gefüllt und durch Hinzufügen von Phosphatpuffer und Wasser auf das entsprechende pH und die nötige Salzkonzentration gebracht. Dann wird dem Gemisch Thrombinlösung zusetzt (Lösung A). Binnen einigen Minuten vollzieht sich die Umwandlung des Fibrinogens. Das nach weiteren 10—15 Minuten entstandene Fibrin ist mittels Spatel von der Rörchenwand leicht ablösbar und zu einer flachen Masse knetbar. Nach mehrmaligem gründlichen Waschen mit NaCl-Lösung und danach mit Wasser trocknet man das Fibrin in den Röhrchen und wägt. Bei der Bestimmung des Fibrinogengehalts des Oxalatplasmas wird zwecks Erreichung des entsprechenden pH 1 ml Plasma 1 ml M/5 primäres K-Phosphat und 1 ml Wasser zugesetzt. Im Falle der Fibrinogenlösung in NaCl fügt man 1 ml Lösung 1 ml M/5 Phosphatpuffer (primär : sekundär = 3 : 1) und Wasser zu. Den Lösungen, in denen das Fibrinogen sich in einem Phosphatpuffer befindet, wird den zu bestimmenden 1 ml Fibrinogenlösung primäres Phosphat und 1 ml Wasser zugegeben. Bei solchen Zusammenstellungen löst sich das Fibrin von der Gefässchenwand leicht ab und kann mit einer Spatel zu einer flachen Masse gedrückt werden. Die Daten der Tabelle zeigen die auf diese Weise entstandenen und gewogenen Fibrinmengen. Das in den Lösungen befindliche Gesamteiweiss wird mittels warmer Trichloressigsäure ausgefällt und sein Gewicht

nach gründlichem Waschen des Niederschlags mit Wasser und darauffolgendem Trocknen in den tarierten Rörchen bestimmt.

Thrombinherstellung.

Das für die Umwandlung von Fibrinogen in Fibrin verwandte Thrombin wurde analog der oben besprochenen Schweineplasmaherstellung aus Rinderoxalatplasma gewonnen. Bei der Thrombinherstellung verfuhr ich im wesentlichen nach der Methode von Astrup.[2] Ich goss 1 l Plasma in die 10fache Menge angesäuerten dest. Wassers; der Niederschlag setzte sich bei 0° C über Nacht ab. Nach dem Zentrifugieren löste ich diesen in calciumfreier Ringerscher Lösung (300 ml) auf und liess ihn nach Hinzufügung von $CaCl_2$ und Hirnextrakt (s. Astrup) gerinnen. Nach Auspressen und Entfernung des entstandenen Fibrins diente die auf diese Weise gewonnene Lösung bei einem grossen Teil der Versuche als Thrombinlösung (Lösung A). Wird in einer solchen Thrombinlösung mittels 2 Vol. Aceton bei Zimmertemperatur ein Niederschlag erzeugt und dieser abzentrifugiert, sodann aus diesem mit 80 ml einer 0,7%iger NaCl-Lösung das Thrombin herausgelöst, so kann eine etwa 10fache Reinigung erzielt werden. Dialysiert man die auf solche Art gewonnene gereinigte Lösung gegen dest. Wasser, dann entsteht ein Niederschlag, der sich in einigen ml Ringerscher Lösung gut löst und starke Thrombinaktivität aufweist (Lösung B), Fibrinogen jedoch nicht allein zum Gerinnen bringt, vielmehr solches auch auflöst.

Kinetik der Thrombinwirkung.

Zeitlicher Verlauf der Reaktion. Mittels Zugabe von Kaliumpermanganat kann die Fibrinogenumwandlung zu jedem Zeitpunkte zum Stillstand gebracht werden. Das bis dahin enstandene Fibrin kann auf die oben beschriebene Weise von dem unveränderten Fibrinogen getrennt und getrocknet abgewogen werden. An Hand dieses einfachen Verfahrens kann auch der zeitliche Verlauf der Reaktion verfolgt werden. Nachstehender Versuch bezweckt eine solche Untersuchung: In abgewogene Zentrifugenrörchen füllte ich 2 ml Fibrinogenlösung (enthaltend 9,2 mg Fibrinogen) und 1 ml dest. Wasser. Nach

dem Hinzufügen und Vermischen mit 0,3 ml 3fach verdünnter Thrombinlösung (Lösung A) stellte ich mittels 0,5 ml N/10 Kaliumpermanganatlösung die Reaktion zu verschiedenen Zeitpunkten ab. Nach dem Zusetzen des Permanganats drückte ich das entstandene Fibrin zusammen, währenddessen sich das Permanganat mit der Flüssigkeit vermischt, und die Reaktion zum Stillstand kam. Die Flüssigkeit wurde vom Fibrin abpipettiert, das Fibrin auf der bereits erwähnte Weise gewaschen, getrocknet und gewogen. Das Abstellen der Reaktion ist

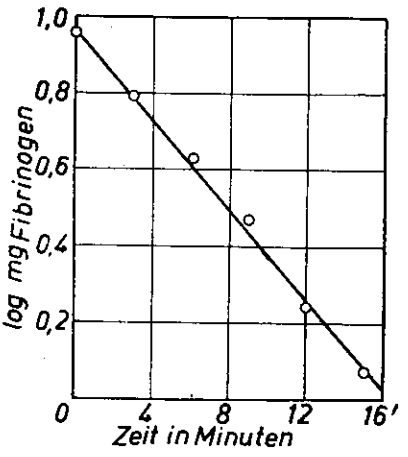


Abb. 1.

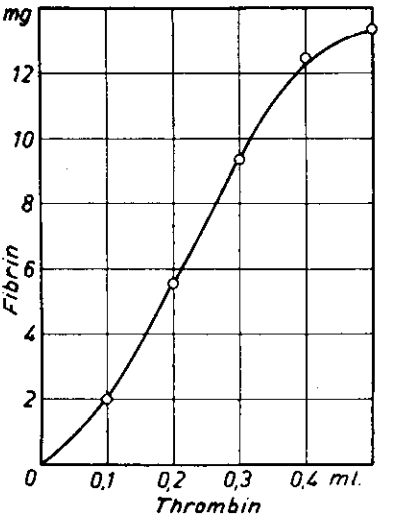


Abb. 2.

auf diese Weise binnen 15—20 Sekunden durchführbar. Abb. 1 zeigt das Versuchsergebnis: auf die Ordinate sind die Logarithmen der nicht umgewandelten Fibrinogenmengen in mg aufgetragen, während die Abszisse die bis zum Abstellen mittels Permanganat verstrichene Reaktionszeit angibt. Wie auf der Abbildung ersichtlich, scheint die Fbrinogenumwandlung, oder zumindest die Fibrinausscheidung — wie die Eiweissdenaturierungsreaktionen im allgemeinen — den monomolekularen Reaktionsstypus zu befolgen.

Einfluss der Thrombinmenge auf die Reaktion. Abb. 2 zeigt, wieviel Fibrin von verschiedenen Thrombinmengen aus der Fibrinogenlösung in gleichen Zeiten hergestellt werden. Der Versuch war in folgender Weise zusammengestellt. Ich beschickte auf die vorhergehende Weise kleine Zent-

rifugierröhrchen mit 1 ml Fibrinogenlösung (enthaltend 16,6 mg Fibrinogen), 1 ml M/5 primärem Phosphat und 1,5 ml dest. Wasser und mit wechselnden Mengen Thrombin; in das eine Röhrchen kam 0,5 ml Thrombinlösung (Lösung A), in das andre 0,4 ml Thrombinlösung + 0,1 ml Ringersche Lösung usw. Nach dem Hinzufügen der Thrombinlösurg stellte ich die Reaktion nach 13 minuten mittels 1 ml der beschriebenen Permanganatlösung ab. Auf der Abbildung stellt die Ordinate das entstandene Fibrin in mg, die Abszisse die Thrombinmengen dar. Die ergebnisse zeigen, dass die entstandene Fibrin-

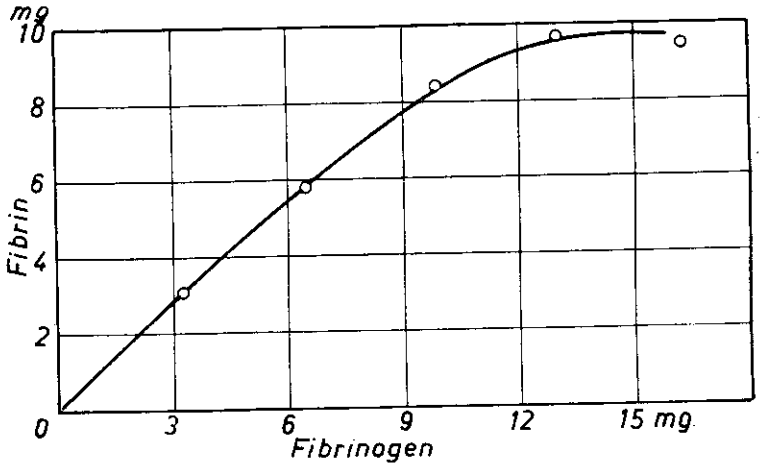


Abb. 3.

menge bis zu einer gewissen Grenze in geradem Verhältnis zu der zugefügten Thrombinmenge steht.

Einfluss der Fibrinogenmenge auf die Reaktion. Ähnlich dem vorhergehenden Versuche zeigt Abb. 3 wieviel Fibrin aus verschiedenen Fibrinogenmengen auf die Wirkung gleicher Thrombinmengen hin entsteht. Ich pipettierte in kleine Zentrifugierröhrchen 1 ml (enthaltend 16,3 mg Fibrinogen) und 0,8, 0,6, 0,4 ml Fibrinogenlösung und füllte diese in allen Gefässen mit Phosphatpufferlösung auf 1 ml auf. Nach Hinzufügen von 1 ml M/5 primären Phosphat und 1 ml dest. Wasser wurde dem Gemisch 0,2 ml Thrombinlösung (Lösung A) zugesetzt. Jedes Röhrchen wurde 5 Minuten nach dem Beimischen von Thrombinlösung mittels Permanganat versetzt.

Auf der Abbildung zeigt die Ordinate das entstandene Fibrin in mg, die Abszisse die Fibrinogenausgangsmengen in mg.

Alle diese Versusche weisen auf das enzymartige Verhalten des Thrombins hin, allein die endgültige Entscheidung der Frage ist ohne Isolierung des Thrombins nicht möglich.

Fibrinolyse. Die fibrinlösende Wirkung von Thrombinpräparaten wird von nachstehendem Versuche veranschaulicht. Ein kleines Zentrifugierröhrchen wird mit 0,3 ml Fibrinogenlösung (4 mg Fibrinogen enthaltend), 0,3 ml M/5 primären Phosphat, 0,6 ml dest. Wasser und 0,5 ml gereinigter und konzentrierter Thrombinlösung (Lösung B) beschickt. Das Fibrinogen gerinnt binnen einigen Sekunden. Setzt man nunmehr dieses, das geronnene Fibrinogen enthaltende Röhrchen in ein Wasserbad von 45° C, dann löst sich das Fibrin binnen 1—1½ Stunden vollständig auf. Das gelöste Fibrin koaguliert nach dem Zusetzen weitern Thrombins nicht aufs Neue. Scheinbar ist diese lösende Wirkung des Thrombins ganz spezifisch für Fibrin, denn bringt man die Fibrinogenlösung obiger Zusammensetzung durch Wärme zur Gerinnung, dann löst sich das gefällte Fibrinogen durch Thrombin nicht auf. In gleicher Weise bleibt auch das durch Trichloressigsäure gefällte und gewaschene Fibrinogen unlöslich.

Die Absorptionsspektren von Fibrinogen und Fibrin.

Die Fibrinogenlösung gerinnt bei pH 6,2 auf die Wirkung von Thrombin hin zu einer undurchsichtigen weissen Masse. Bei pH 7,2 bleibt Fibrin in M/5 Phosphatpufferlösung vollkommen durchscheinend, nur eine geringe Zunahme der Trübung ist zu beobachten. Unter solchen Umständen ist auch das Ultraviolettabsorptionsspektrum des Fibrins messbar. Das Absorptionsspektrum des Fibrinogens und des Fibrins wurde von P *Csokán* aufgenommen.[3] Die Versuche erwiesen, dass das Fibrinogenspektrum im allgemeinen die Eigenschaften der Eiweisspektra aufweist: einen Absorptionsstreifen bei $279\mu\mu$ und eine Beugung bei $255\mu\mu$. Das Fibrinspektrum zeigt nebst den in Fibrinogen erscheinenden Streifen auch das Auftreten eines neuern Streifens: einen niedrigen solchen um $400\mu\mu$ herum. Aus dem Auftreten dieses neuen Maximums kann man bezüglich der

Struktur des Fibrins einen interessanten Schluss ableiten. Nach KISS u. MITARB.[4] tritt bei den Schiffschen Basen vom Typus *a* unserer Abb. 4 in Fällen, in denen der Stickstoff der Azomethingruppe (— H C=N —) durch Vermittlung einer Wasserstoffbindung an eine OH-Gruppe gebunden ist, neben den Streifen der Azomethingruppe ein neuerer Streifen auf; als Reaktion der durch die Wasserstoffbindung gestörten Azomethingruppe erscheint dieser Streifen genau auf derselben Stelle wie der oben erwähnte neue Streifen des Fibrins.

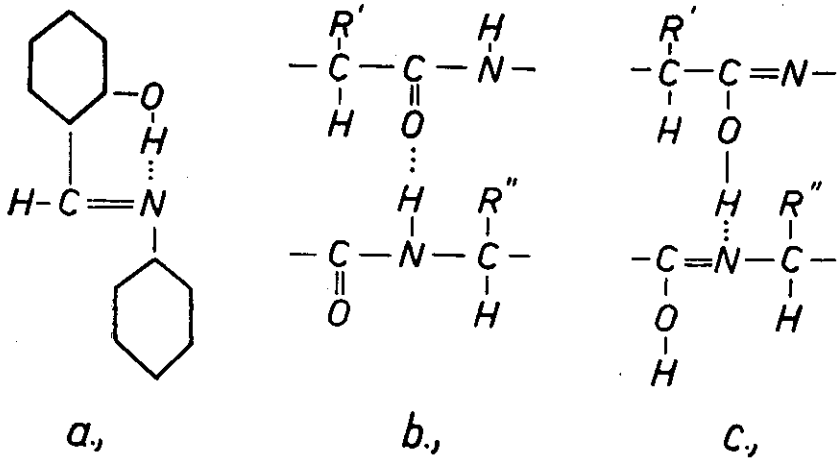
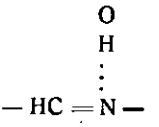


Abb. 4.

Auf der Abbildung 4. zeigt die Formel *b* zwei Polypeptidkettenstücke, wie sie sich im Eiweissmolekül mittels Wasserstoffbindungen verbinden. Nach den Infrarotabsorptionsuntersuchungen von RODEBUSH u. MITARB.[5] kommt diese Wasserstoffbindung zwischen der NH-Gruppe und dem Carbonyl zustande.

Ordnet man die Doppelbindungen nach Formel *c* um, dann erhält man in der Polypeptidkette genau die gleiche Struktur bzw. Umlagerung, die nach BERGMANN[6] auf der Oberfläche der peptidspaltenden Enzyme zustande kommt. Die dergestalt umgebildete Formel enthält zugleich genau dieselbe Atomgruppierung, die bei den vorhin erwähnten Schiffschen Basen den auf das Auftreten der Wasserstoffbindung hinweisenden neuen Streifen hervorbringt.



Die Analogie würde, soweit sie zutreffend ist, zeigen, dass beim Unwandeln des Fibrinogens in Fibrin diese Umlagerung der Doppelbindungen stattfindet. Als Folge der Umlagerung würde mit den in die Polypeptidkette geratenen Doppelbindungen einigermassen eine Versteifung sowie eine Verkürzung der Kette einhergehen. Nach den Röntgenuntersuchungen von KATZ u. ROOY[7] ist die Identitätsperiode im orientierten Fibrinfaden entlang dem Faden 6,7 Å anstatt 7,0 Å.

Zusammenfassung.

In gereinigten Fibrinogenlösungen bewirkt Thrombin eine vollständige Umwandlung des Fibrinogens in Fibrin. Die Umwandlung scheint eine monomolekulare Reaktionstypus zu befolgen. „Fibrinolysin" macht einen scharfen Unterschied zwischen Fibrin und wärmedenaturiertem Fibrinogen. Das Ultraviolettabsorptionsspektrum des Fibrins zeigt neben den Absorptionsstreifen des Fibrinogens auch einen neuern Streifen, der auf das Auftreten von Wasserstoffbindungen von gewissem Typus hinweist.

Literatur.

1. *K. Laki,* Z. physiol. Chem., *273,* 95, (1942).
2. *T. Astrup, S. Darling,* J. Biol. Chem., *133,* 761, (1940).
3. *P. Csokán, K. Laki.* Z. physik. Chem., A. *190,* 278 (1942).
4. *Á. v. Kiss, G. Auer,* Z. physik. Chem., A. *189,* 344 (1941).
5. *A. M. Buswell, I. R. Downing, W. H. Rodebush,* J. Amer. Chem. Soc., *62,* 27559, (1940).
6. *M. Bergmann, L. Zervas, J. S. Fruton, F. Schneider, H. Schleich,* J. Biol. Chem., *109,* 325, (1935).
7. *I. R. Katz, A. de Rooy,* Naturwiss., *21,* 559 (1933).

The effect of salts on the isoelectric point of casein.

by

T. Erdős.

(Chinoin research fellow).

It has been described by MICHAELIS and SZENT-GYÖRGYI[1] that small concentrations of neutral salts shift the pH range, in which casein is insoluble, either to the acid or to the alkaline side. I have attempted a more detailed study of this phenomenon.

Hammarsten's casein (Merck) was used in the experiments. A stock solution was prepared by dissolving 0.1 g casein in 100 ml of a 0,004 M Na-acetate solution. The buffer solutions used were acetic acid - Na-acetate mixtures, covering the pH range of 3,0 — 6,0 the difference between each two members of the series being 0,05 pH units.

The experiments were made in the following way: 1 ml of the 0,1% casein solution was given to 8 ml of buffer and made up to 10 ml with distilled water. The contents of the tubes were mixed thoroughly, left to stand at room temperature (21—23°) for 24 hours, and the results noted. If sedimentation or visible flocculation was observed, the result was marked „precipitated", if there was only opalescence but no flocculation, it was marked „not precipitated". After reading the results, the pH of the solutions was controlled electrometrically.

It is seen from Table I that an increase in the concentration of the buffer will shift the precipitation zone to the acid side. There is no difference between the effect of 0,02 and 0,01 M buffers. In these buffer solutions casein is precipitated between pH 4,32—5,00. The middle of this range, pH 4,66, agrees well with the known values of the isoelectric point of casein:

Table I.

M concentration of the buffer	Range of precipitation
1.00	3.70 — 4.70
0.50	3.92 — 4.78
0.20	4.15 — 4.85
0.10	4.20 — 4.90
0.04	4.27 — 4.96
0.02	4.32 — 5.00
0.01	4.32 — 5.00

pH 4,7 according to RONA and MICHAELIS,[2] 4,6 according to MICHAELIS and PECHSTEIN.[3]

In the following experiments I have used a 0,02 M buffer to adjust the pH of the casein solutions. In certain cases, where a higher concentration of the buffer was needed, I have made allowance for the modifying action of the buffer concentration.

1 ml of a 1,0 M salt solution or the corresponding amount of salt in substance was added to 8 ml acetate buffer and 1 ml 0,1% casein. The volume was made up to 10 ml with dist. water. After standing at room temperature for 24 hours the precipitation was noted. In Fig. 1. the results of these experiments are summarized. The horizontal lines indicate the pH-range in which casein was precipitated. The salt added to the buffered casein is indicated by its formula over the precipitation zone. The final concentration of these added salts was 0,1 M except in the case of CH_3COOAg, whose final concentration was 0,05 M and $AgNO_3$ which was studied in both of these concentrations. Whereas in most cases the concentration of the buffer was 0,02 M, in some cases the concentration was 0,1 M and in others the buffer was a 0,5 M acetate buffer. In these cases allowance was made for the shift in the precipitation zone due to the buffer and this being substracted the net effect of the salt is shown in Fig. 1. In the case of Na_2HPO_4 there is no acetate buffer, but phosphoric acid was added to give the desired pH. The horizontal line marked „0" shows the precipitation pH-range of casein in 0,02 M acetate buffer.

Special attention was paid to the effect of the alkali haloids.

It is seen at first sight from Fig. 1. that most of the salts shift the precipitation zone towards the acid side. This shift is obviously due to the anion, its extent being the function of the atomic weight. Cations have a pronounced secondary

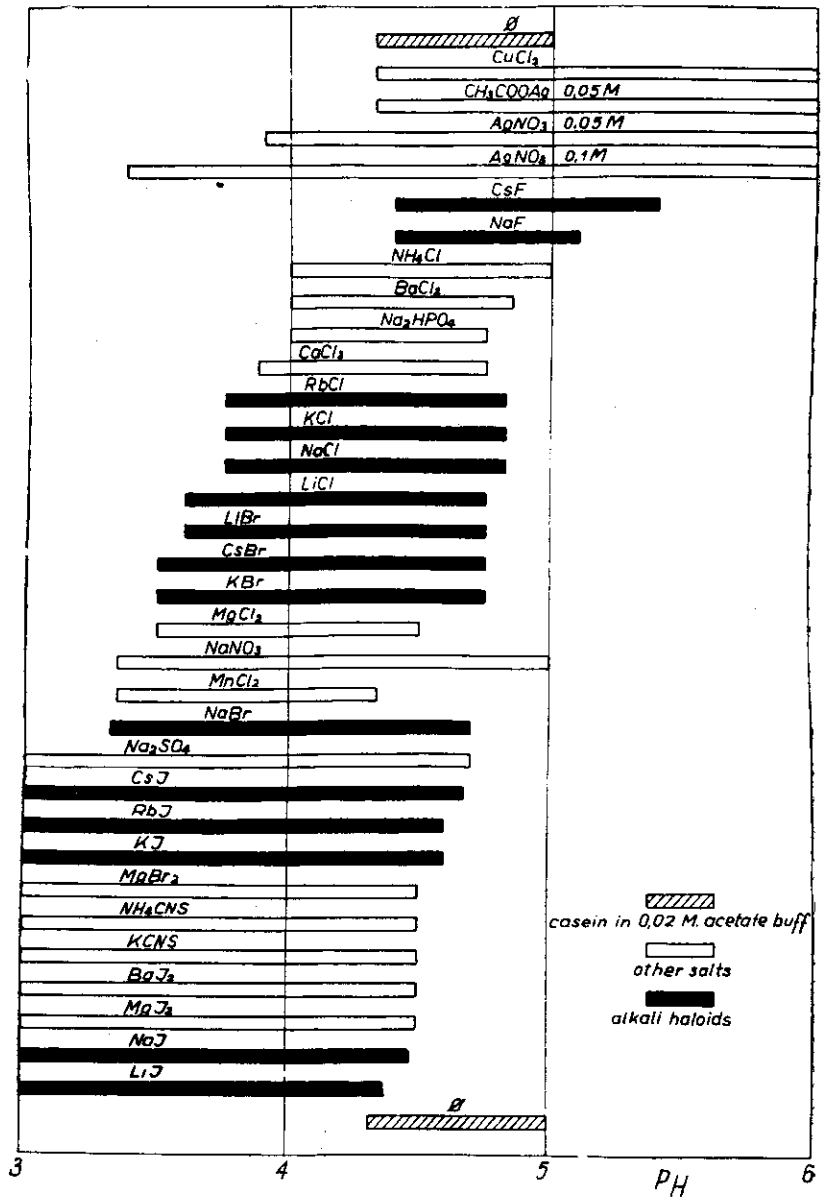


Fig. 1.

influence, shifting the precipitation zone towards the alkaline side. In this case too, the effect is proportional to the atomic weight. In two cases (the two fluorides) the precipitation zone is shifted altogether towards the alkaline side, showing that in these salts the cations take the upper hand. The iodides have a remarkable precipitating action, they would precipitate casein even at pH 1 (not shown in the figure). Similar results have been obtained with the Mg, Ca and Ba haloids. LiBr had exceptional influence, the effect of this salt would not fit into the system.

We may draw the conclusion that the effect of a salt results from the effect of its anion and cation. If the effect of its anion is stronger, the precipitation zone will be shifted to the acid side. If the effect of the cation is stronger the reverse will happen. On the whole the results confirm the data of MICHAELIS and SZENT-GYÖRGYI.

The precipitations obtained at these different pH values in presence of salts are all reversible and do not change the properties of casein. This is demonstrated by the following experiment. The precipitate obtained at pH 3,0 in presence of NaJ was centrifuged off and the small volume of precipitate dissolved in a 0,02 M acetate buffer: its precipitation zone was now identical with the original precipitation zone of casein. i. e. 4,32—5,00.

Next I have studied the influence of the salts on the acid-base binding capacity of casein. Two salts, NaCl and NaJ have been chosen for these experiments, the former giving a small effect, the latter a strong effect in the precipitation experiments. It was found that both salts have identical effects, both of them increase the acid-base binding capacity to the same extent and neither of them changes the isoionic point of casein.

First I made an electrometric titration curve by adding 0,1 N HCl to a 0,01 N NaOH solution and a similar curve by adding 0,1 N NaOH to a 0,01 N HCl solution. 1% casein solutions were made in 0,01 N NaOH and 0,01 N HCl, and again the electrometric titration curve was made by adding different quantities of NaOH or HCl. The titration curve was naturally different of the titration curve of the pure acid or base owing to the acid-base binding capacity of the casein. The difference of the two curves gives the acid-base binding capacity, the

minimum of which (i. e. the isoionic point) was in my experiments at pH 4,6 which agrees well with the accepted value in the literature. Then the experiments have been repeated in the presence of 0,1 and 1,0 M NaCl and NaJ respectively. The acid-base binding capacity was increased by the presence of either salt the more, the higher the concentration of the salt was in the solution. There was however no difference between the

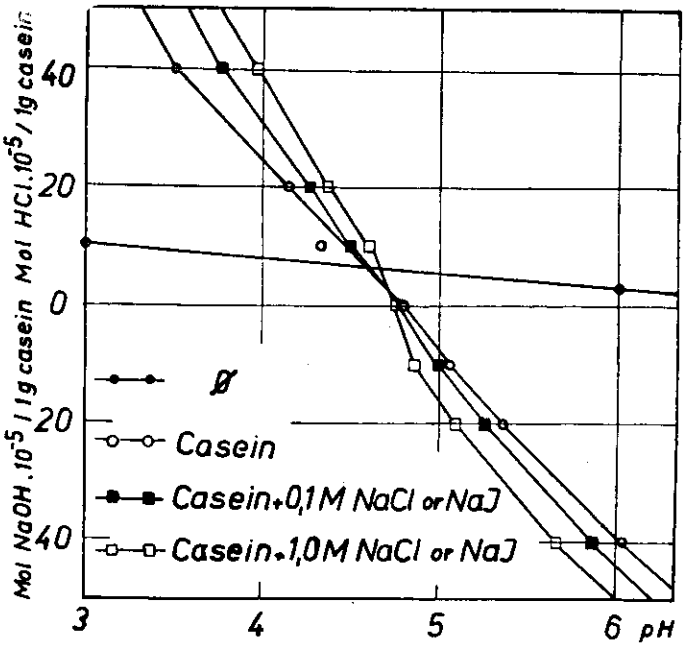


Fig. 2.

effect of NaCl and NaJ. The isoionic point was not changed by the presence of the salts. (Fig. 2.)

We may conclude therefore that the change in the isoelectric pH range of casein due to the presence of different salts in the solution cannot be explained by assuming an effect of the salts on the acid-base binding capacity of casein.

Literature.

1. *L. Michaelis* u. *A. Szent-Györgyi*, Biochem. Z., *103*, 178 (1920).
2. *P. Rona* u. *L. Michaelis*, Biochem. Z., *28*, 193 (1910).
3. *L. Michaelis* u. *H. Pechstein*, Biochem. Z., *47*, 260 (1912).

On the electronegativity of atoms and their influence on the isoelectric point of casein.

by

K. Laki.

The electric charge of a protein particle in absence of salts is determined by the pH of the solution. At pH below the isoelectric point the protein particle takes up hydrogen ions in excess and aquires hereby a positive charge. At the opposite side of the isoelectric point the protein particle aquires a negative charge the hydroxyl ions being in excess. The quantity of the charge taken up by the protein particle increases as we depart from the isoelectric point. The charge has its maximum at a pH where the protein particle reaches the maximum of its acid or base combining capacity.

Curve A in figure 1. shows schematically how the acid combining capacity of casein (Hammarsten) changes with the pH, the shape of the curve being determined from the data of titrations combined with pH measurements. The curve reaches the abscissa at pH 4,8 (the isoelectric point of the casein) and becomes assymptotic at pH 2 showing that the protein reached here the maximum of its acid combining capacity and also the maximum of the positive charge that can be obtained.

If, at a pH where the casein particle has a positive charge, we add a salt which neutralises this charge,[1] then the casein particle will have no charge in excess and will have its isoelectric point at this pH. If this neutralisation happened at a pH at which the charge of the casein is not yet maximal, on further acidification the protein particle becomes positively charged again. Curve B in figure 1. gives an example of this case. The charge of the casein is neutralised at pH 4 by the addition of a salt. On further acidification the casein particle takes up a positive charge again. It is clear that if we neutra-

lise the charge of the casein at a pH where the charge maximum has already been reached (pH 2), on further acidification it aquires no charge again. It follows that in presence of salt the isoelectric point depends on the extent to which this salt can neutralise the charge of the casein particle. On the pH scale the isoelectric point becomes thus displaced from its original position. This displacement is a measure of the electric charge by which the salt neutralises the casein particle.

At the isoelectric point the casein precipitates from the

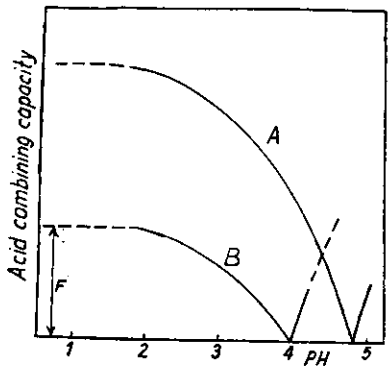


Fig. 1.

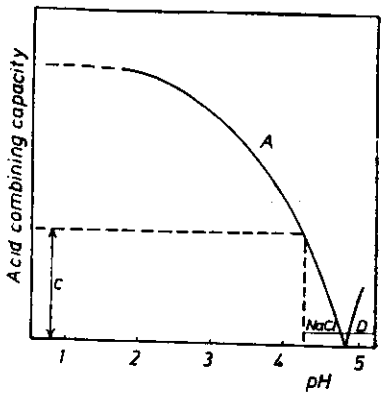


Fig. 2.

solution, and also precipitates in the neighbourhood of it indicating that the casein particle needs a certain quantity of charge to remain in solution. Correspondingly the casein has, instead of a point, a zone of precipitation on the pH scale.

Some precipitation zones, obtained in the presence of halogen salts of alkali metals, can be seen on fig. 3 taken from the preceding paper (Erdős). Strict comparison of the single zones is impossible because the left end of some of these zones is not defined, and so the exact position of the isoelectric point can not be given (calling the middle of the zones the isoelectric point).

The fact that in case of certain salts only the right end of these zones is defined can be explained in the following way. In figure 2 the line D represents the precipitation zone of the casein in the presence of NaCl. It can be seen from the figure that the casein remaines precipitated in a fairly large

pH zone until its charge exceeds a certain quantity (C) which is necessery to bring the casein into solution. Now let us suppose that a certain salt displaces the zone and the isoelectric point. In this case curve B in fig. 1. shows how the casein can be charged again by altering the pH. The comparison of the charge quantities (C and F in fig. 2. and 1.) shows that the amount of charge in this case is insufficient even at pH 2 to charge the casein properly and to bring it into solution.

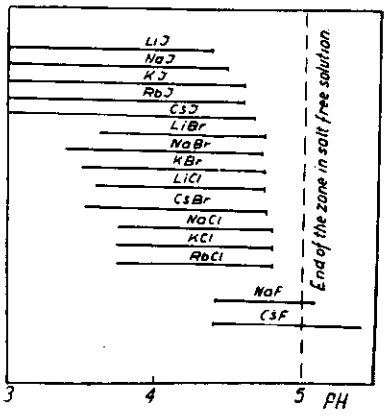


Fig. 3.

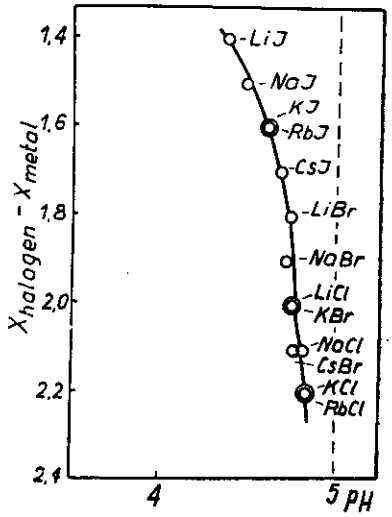


Fig. 4.

The casein thus would remain undissolved throughout the whole acid pH range. It follows from these that in cases where the precipitation zones are displaced to some extent, the zones will extend to cover the whole acide pH range having no defined end to the left. This is the case e. g. with NaJ and LiJ in fig. 3.

Since the left end of the zones in many cases is not defined the middle of the zones can not be ascertained and given as the isoelectric point. We are thus confined to the defined right end of the zones if we want to use them as a basis for comparison.

In figure 4. the circles represent the right ends of the precipitation zones of fig. 3. The abscissa gives the pH scale, the ordinata the values of electronegativity differences of the atoms composing the salt. It can be seen that the displacement

of the precipitation zones is correlated to the values of electronegativity differences.

The electronegativity represents the power of an atom in a molecule to attract electrons,[2] and MULLICAN[3] pointed out that the average of the first ionisation energy and the electronaffinity of an atom can be the measure of its electronegativity.

This correlation of electronegativity values with the power of salts to displace the isoelectric point suggests that the ionisation energy and electronaffinity of atoms play a role in the chargeing process in which the charge of the casein particle is neutralised.

In the following discussion an attempt will be made to explain — at least qualitatively — how the electronegativity differences can be brought into connection with the charge given to the protein particle.

In the past few years a new general theory of the solid matter has been developing.[4] According to this theory the solid matter can be regarded as a large molecule containing a great number of atoms which are arranged in a fashion which gives a regular lattice system. Because of this regular arrangement the valence electrons of these atoms belong to the whole system of atoms having common energy bands. This theory has been successful in interpreting many of the various properties of solids.

There is an increasing number of observations about the behaviour of the protein molecules which can be explained by picturing the protein praticle as a piece of solid matter[5] built up by C, N, O, H atoms. These atoms or a part of them are arranged in such a manner as to give a regular lattice at least in one dimension. The H atom in the hydrogen bond plays an important part in building up the lattice.

In the following treatment this new theory of solid matter will be accepted for the casein particle and discussed what sort of changes occur when a new atom is brought to the surface of the particle.

Suppose, that an atom such as Na having a low ionisation energy is brought on the surface of the casein particle the lattice atoms of which have a higher ionisation energy. Then it might happen that the electron of the Na atom leaves its core and enters into the continuum built up by the atoms

of the casein particle. The result of this is that the Na will be ionised and positively charged and the lattice atom nearest to the surface negatively charged.

In a case where the difference between the ionisation energies is small, the chargeing of the casein also may occur if the lattice atom has a greater electron affinity compared with that of the adsorbed atom. Then the lattice atom attracts an electron from the adsorbed atom, thus the surface atom of the casein will have a negative charge, the adsorbed atom a positive charge.

It follows therefore that two factors determine the magnitude of the charge given to the surface of the casein particle by the adsorption of a foreign atom: 1) the difference between the ionisation energies and 2) the difference between the electron affinities of the adsorbed atom and the lattice atoms.

These two factors can be symbolised in the following way: 1.) $J_l - J_a$ symbolises the magnitude of the charge caused by the difference in the ionisation energies of the adsorbed atom (J_a) and the lattice atom (J_l). 2.) $E_l - E_a$ represents the magnitude of the charge caused by the difference of the electron affinities of the lattice (E_l) and adsorbed (E_a) atom.

Adding up these two symbols we get the following expression:

$$J_l - J_a + E_l - E_a = J_l + E_l - (J_a + E_a)$$

$J_l + E_l - (J_a + E_a)$ represents the total charge given to the casein molecule.

The sum of $J + E$ (ionisation enregy and electron affinity) devided by 130 is identical with the electronegativity values (X) of the atoms.[2] Taking this into consideration the charge given to the casein particle can be represented by the differences of the electronegativities of the adsorbed (X_a) and the lattice composing (X_l) atoms: $X_l - X_a$.

Now if a Cl atom is brought to the surface of the casein particle and this atom behaves in the opposite way as the Na atom (it has a higher ionisation energy and greater electron-affinity than the lattice atom,) the magnitude of the charge given to the casein particle can be represented by the following symbole: $X_b - X_l$. Where X_l means the electronegativity of the lattice atom and X_b represents the electronegativity of the adsorbed Cl atom.

If both Na and Cl atoms are simultaneously on the surface, the resulting charge can be given by adding up these two expressions:

$$X_i - X_a + X_b - X_i = X_b - X_a$$

The symboles representing the electronegativities of the lattice atoms fall out and there remains the difference of the electronegativities of the Cl and the Na atoms: $X_b - X_a$.

The Na and Cl atoms according to this picture are ionised on the surface as Na and Cl ions.

The symbole $X_b - X_a$ says that the magnitude of the charge given to the casein particle is related to the values of the electronegativity differences of the salt composing atoms, and so gives the same result which is born out by the experiment.

Literature.

1. *Takeo Ito,* Biochem. Z., *233,* 444, 1931.

2. *L. Pauling,* The nature of the Chemical bond. (Cornell Univ. Press, 1939.)

3. *R. S. Mulliken,* J. Chem. Phys., *2,* 782, 1934., *3,* 573, 1935.

4. *F. Seitz, R. P. Johnson,* J. Applied Phys., *8,* 84, 1937., *8,* 186, 1937., *8,* 246, 1937.

F. Seitz, The modern theory of solids. (McGraw-Hill Book Comp. 1940.)

5. *F. Möglich* a. *M. Schön,* Naturwiss., *26,* 199, 1938.

P. Jordan, Naturwiss., *26,* 693, 1938.

N. Riehl, Naturwiss., *28,* 601, 1940.

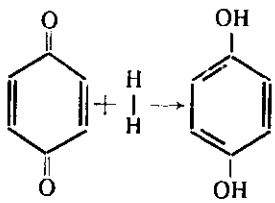
N. Riehl, N. W. Timoféeff-Ressovsky u. *K. G. Zimmer.* Naturwiss., *29,* 625, 1941.

Redoxpotential and resonance energy of certain quinones.

by

K. Laki.

The reaction which takes place when a quinone is hydrated to the corresponding hydroquinone is symbolised by the following equation:



The energy change accompanying this reaction can be calculated from bond energy data. According to the above equation one H—H, two C=O, two C=C and four C—C bonds must be broken and two O—H, two C—O, three C=C and three C—C bonds must be rebuilt. Since in all reactions in which a paraquinone is hydrated to hydroquinone the same structural change occurs, the energy change calculated from bond energies will have the same value. Correspondingly the redoxpotential calculated from this value would have the same value in all cases. (The entropy in these reactions has a standard value[1].) Various quinone-hydroquinone systems however have different redoxpontential values. The resonance energies are responsible for this fact. These energy values must be included into calculations useing bond energy data.

In the following table some data are given to show how the resonance energy values are related to the redoxpotential values of some quinone-hydroquinone redox-systems. In col. 1. the redoxpotential values[2], in col. 2. the resonance energy

values of quinones and hydroquinones, and in col. 3 the difference of these resonance energy values are given. On comparing col. 1. and 3 it can be seen that the redoxpotential becomes smaller and smaller as the difference of the resonance energies between quinones and hydroquinones diminishes. Thus the redoxpotential seems to be a function of the resonance energy.

Table.

	Redoxpot. in Volts	Resonance energy in kcal./mole	Difference of resonance energies.
Quinone	0,681	— 3.3	51,0
Hydro „		47,7	
Toluquinone	0,623	5,4	45,0
Hydro „		51,3	
Thymoquinone	0,579	6,0	43,0
Hydro „		49,0	
Naphtoquinone	0,492	47,5	41,5
Hydro „		89,0	
Antraquinone	0,155	99,0	23,0
Hydro „		122,0	

The values of resonance energies given in col. 2. were calculated from thermochemical data comparing the values of heat of formation with the values of the sum of bond energies. Most of the bond energies were taken from the book of L. PAULING.[3] For the energy of the C—O bond a 20% higher value[4] was used than that given by PAULING. The C = O bond energy value used in these calculations was 16 kcal higher than that given for ketons. This seemed justified since the distance of the C = O bond in quinones[5] and the CO_2 molecule[6] is nearly the same.

Literature.

1. *G. Holst*, Z. physical. Chem., *A 183*, 423, 1939.

2. *J. Conant, L. Fieser*, J. Am. Chem. Soc., *45*, 2194, 1923., *46*, 1858, 1924.

3. *L. Pauling*, Nature of the Chemical Bond. (Cornell Univ. Press, 1939.)

4. *K. W. F. Kohlrausch*, Ber. *69 B*, 527, 1936.

5. *J. M. Robertson*, Proc. Roy. Soc., *A 150*, 106, 1935.,
Bwagawati Charan Guha, Phil. Mag., *26*, 213, 1938.

6. *W. V. Houston* and *C. M. Lewis*, Phys. Rew., *38*, 1827, 1931.

Das Oxydationsferment des Kartoffelgewebes.

von

W. F. H. M. Mommaerts.*

Die Oxydationssysteme der höheren Pflanzen sind nur noch sehr unvollkommen bekannt. Es gibt Systeme die durch Vorherrschen von Peroxydase, Askorbinsäureoxydase, oder Phenoloxydase gekennzeichnet sind (1), während neuere Arbeiten auf die Bedeutung der Cytochrome und der Cytochromoxydase hinweisen (2, 3; vergl. auch schon Keilin 4). Keines dieser Systeme ist eingehend erforscht. Es wurde daher das Studium der Kartoffelatmung, als Beispiel einer Pflanzenatmung, vorgenommen

Kartoffeln enthalten bekanntlich eine hochaktive Phenoloxydase, die von Kubowitz (5) als ein Kupferproteid erkannt wurde. Die Deutung der physiologischen Rolle des Ferments ist schwer: im zerkleinerten Gewebe findet die Sauerstoffaufnahme 15 bis 20 mal schneller statt wie in Gewebeschnitten (6); (in diesen Versuchen mit Gewebebrei wurde das bei der Oxydation des Katechinderivats gebildete Chinon mittels Askorbinsäure reduziert.) Dieser Befund zeigt dasz die Oxydase, dem reduzierenden System gegenüber, in Überschusz anwesend ist; man würde also erwarten dasz in der Zelle das gesamte Katechinderivat als Chinon vorliegen würde; dies trifft aber nicht zu, weil dann das Gewebe sich braunfärben würde, und die Oxydase gehemmt sein sollte. (Vergl. 7, 8) Zur Erklärung dieser Diskrepanz hat man angenommen dasz das Ferment in der Zelle in inaktiver Form vorliege, oder dasz in vivo Ferment und Substrat getrennt seien.

Um zwischen diese beide Möglichkeiten eine Entscheidung zu treffen habe ich zuerst versucht das Ferment in in-

---

* Stipendiat des „De Groot — Fonds", den Haag.

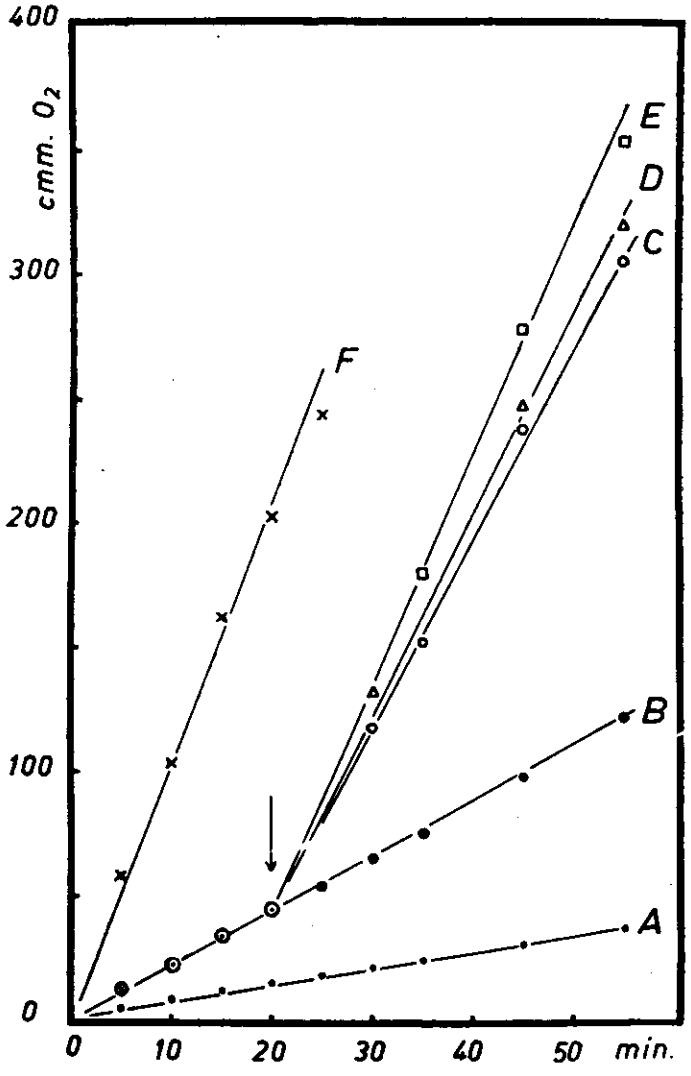


Fig. 1.

A. bis E.: Atmung von 400 mgr. Kartoffelgewebeschnitte in 2.5 cm.³ $\frac{m}{15}$ Phosphatpuffer p_H 6.4 bei 26°.

A: ohne Zusatz; B bis E mit Askorbinsäure in Überschusz; in C, D. und E nach 20 Min. Zugabe von resp. 4.10^{-7}, 2. 10^{-6} und 10^{-5} gr. mol. Brenzkatechin.

F.: Atmung von 400 mgr. Kartoffelbrei in Phosphatpuffer mit Askorbinsäure in Überschusz.

aktiver Form zu extrahieren; dies blieb aber ohne Erfolg. Hingegen sprechen folgende Befunde zugunsten der zweiten Deutung:

Askorbinsäure steigert die Sauerstoffaufnahme von Kartoffelschnitten um mehrere hundert Prozente; wird jetzt überdies noch Brenzkatechin zugegeben, so wird der Sauerstoffverbrauch aufs neue erheblich gesteigert, und zwar bis zu einem Betrag der dem der Gewebebreiatmung (mit Askorbinsäure!) ungefähr gleich ist (Fig. 1.)

Dies spricht für die Annahme dasz in der Zelle das natürliche Brenzkatechinderivat, wenigstens zum grószten Teil, vom Ferment getrennt, und für die Atmung ohne Bedeutung ist. Damit wird natürlich auch die Bedeutung der Phenoloxydase für die Atmung zweifelhaft. Um zu entscheiden ob sich die Phenoloxydase überhaupt an der Atmung beteiligt, habe ich untersucht ob die Atmung und die Phenoloxydase sich in Hemmungsversuchen übereinstimmend verhalten. Um die Aktivität der Phenoloxydase in der Zelle zu messen, kann man nicht einfach die Oxydationsgeschwindigkeit zugegebenen Brenzkatechins bestimmen; das Ferment wird dann nl. schnell inaktiviert, und bei Anwesendheit von Giften werden die Verhältnisse recht undurchsichtig; es wurde daher den Sauerstoffverbrauch nach Zusatz von p-Phenylendiamin mit wenig Brenzkatechin gemessen; das Diamin reduziert das gebildete o-Dichinon, wird aber ohne Katechinzusatz vom Ferment nicht angegriffen.

Es wurde gefunden dasz die Atmung, wie die Phenoloxydase, von Schwermetallantikatalysatoren wie Kohlenmonoxyd, Cyanid, Sulfid, Hydroxylamin, Fluorid, und Azid kräftig bis fast vollständig gehemmt wird. Als Beispiel sei ein Hemmungsversuch mit Hydroxylamin angeführt (Fig. 2.) Hier besteht eine vollständige Übereinstimmung zwischen Atmungshemmung und Hemmung der Phenoloxydase. Nicht immer ist die Übereinstimmung so gut; Abweichungen können von verschiedenen Faktoren verursacht werden n. 1. *1.* Meistens ist das Atmungsferment ungesättigt, weil ein anderes Glied der Fermentkette die Atmungsintensität beschränkt. *2.* Es kann zwischen Katechin und Hemmungssubstanz Konkurrenz um das Fermentkupfer auftreten *3.* Besonders bei höheren Konzentrationen von z. B. Cyanid, Azid und Fluorid kön-

nen auch andere Glieder der Katalysatorkette gehemmt werden.

Die Blausäurehemmung wird durch Kupferionen aufgehoben; Fe, Mn, Zn, Co, und Mg reaktivieren nicht;[1] dies steht in Übereinstimmung mit den Befunden von Kubowitz über die Phenoloxydase. Die Kohlenmonoxydhemmung der Atmung ist

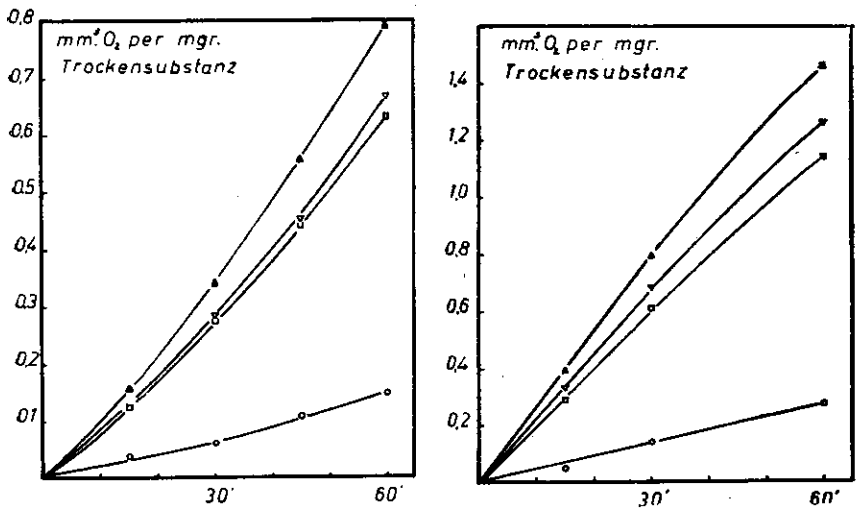


Fig. 2a. Atmung, und Fig. 2b. Phenoloxydaseaktivität unter Einflusz von Hydroxylamin.

▲ Kontrolversuch.
△ Hydroxylamin, 4×10^{-7} gr. mol. per gram Gewebe. (mol/100.)
□ „ 4×10^{-6} „ (mol/1000.)
○ „ 4×10^{-5} „ (mol/10000.)

nicht lichtreversibel, was nach den bisherigen Kenntnissen ebenfalls für Kupferproteide bezeichnend ist.

Starke Atmungshemmungen wurden mit Salicylaldoxim, α-Benzoinoxim, und Natriumdiaethyldithiocarbaminat erhalten. Diese Substanzen reagieren mit Kupfer, z. T. spezifisch, unter Komplexbildung.

---

[1] Auch Ni erhöht die Cyanidgehemmte Atmung, aber nach einem anderen Mechanismus. Während der Kupfereffekt, als eine echte Reaktivierung, bei höheren Konzentrationen des Cyanids und des Metalls immer schwächer ist, nimmt der Nickeleffekt bei höheren Dosierungen zu; offenbar wird durch den Nickel-Cyanidkomplex irgend eine Autoxydation katalysiert.

Die Versuche zeigen also dasz ein Kupferproteid, allem Anschein nach mit der Phenoloxydase identisch, als Atmungsferment des Kartoffelgewebes dient; die Frage welches Substrat dabei primär oxydiert wird, und aus welchen Komponenten das desmolytische System noch besteht, ist der Gegenstand weiterer Arbeit.

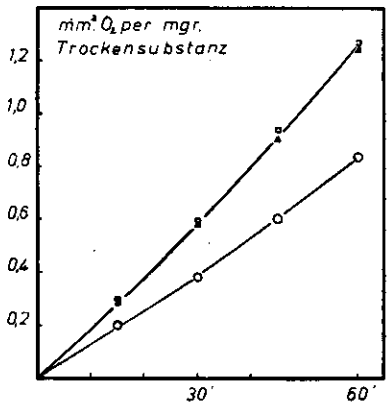


Fig. 3. Hemmung der Atmung durch Cyanid, und Reaktivierung durch Kupfer.

▲ Kontrolversuch.

□ Kaliumcyanid 8×10^{-7} gr. mol. per Gram Gewebe (mol/5000) und $CuCl_2$ 8×10^{-7} gr. mol. per Gram Gewebe.

○ Kaliumcyanid 8×10^{-7} gr. mol. per Gram Gewebe, mit $FeCl_3$, $MnSO_4$, $ZnSO_4$. $Co(NO_3)_2$ und ohne Metallzusatz.

Methodisches. Die Versuche wurden in Warburg-Manometer bei 28,5° C ausgeführt. Es wurde die Atmung von etwa 500 mgr. Kartoffelschnitte (Dicke ungefähr 0,4 mm.) in mol/15 Phosphatpuffer pH 6,4 gemessen. Die Schnitte wurden aus gekühlten Kartoffeln hergestellt und vor dem Versuch 6 Stunden in stehenden Leitungswasser belassen; derartig vorbehandelte Schnitte geben, wie in einer ausführlichen Mitteilung gezeigt werden wird, ein zuverlässiges Bild der Atmung des intakten Gewebes.

Der Hauptraum der Gefässe enthielt die Schnitte in 2 cm.³ Pufferlösung mit etwaige Zusätze; der Einsatz enthielt 0,1 cm.³ KOH 20% (bei Cyanidversuche 2%), die Birne 0,2 cm³ mol/25 p-Phenylendiamin, mol/250 Brenzkatechin, das nach 60 minuten zugegeben wurde.

Literatur.

1. *A. v. Szent-Györgyi,* Studies on Biological Oxydation and some of its Catalysts. Leipzig 1937.

2. *R. Hill & Bhagvat,* Nature *143,* (1939) 726.

3. *P. B. Marsh & D. R. Goddard,* Am. Journ. Bot. 26, (1939) 724—728.

4. *D. Keilin,* Proe. Roy. Soc. *B 98,* (1925) 312.

5. *F. Kubowitz,* Bochem, Z. *292,* (1937) 221—227, *299,* (1939) 32—47.

6. *A. v. Szent-Györgyi & K. Vietorisz,* Biochem. Z. *233,* (1931) 236—239.

7. *D. Richter,* Biochemic. J. 28, (1934) 901—907.

8. *J. G. Boswell & G. C. Whiting,* Annals of Bot. 2, (1938) 846—863.

Aminopherase.

by

P. Lénárd and F. B. Straub.

I.

D. M. NEEDHAM[1] was the first to observe that muscle tissue can decompose l (+) glutamic acid in such a way that the amino group remained in the amino-N fraction. BANGA and SZENT-GYÖRGYI[2] have discovered that glutamic acid reacts with oxaloacetic acid in the presence of muscle tissue. This reaction is so fast and complete that it could be used to trap oxaloacetic acid. The nature of the reaction was not investigated in either of these earlier investigations. It was BRAUNSTEIN and his associates'[3] merit to elucidate the mechanism of the very intensive intermediate metabolism of some natural aminoacids in the muscle. They have shown that a number of aminoacids are able to react with some ketoacids with the exchange of the amino and keto groups. According to these studies such reactions take place in many different organs, the most active among them being skeletal and heart muscle. The significance of these reactions is not yet clear.

No detailed information is available about these reactions from the point of enzyme chemistry. Neither the purification procedures elaborated by BRAUNSTEIN and KRITZMANN,[5] nor that described by COHEN,[4] could bring the question closer to its solution. Studies made on such preparations, assuming that they are purified, proved to be misleading.

We have undertaken the purification of such an enzyme in an attempt to study the mechanism of its action, whether it needs a coenzyme as suggested by BRAUNSTEIN. Moreover, the kinetics of such a reversible reaction between four substrates are of considerable interest from the point of enzyme-

substrate complex formation. Unfortunately, owing to external conditions, this work had to be abandoned at a rather early stage and therefore we decided to describe the purification of the enzyme along with some kinetic measurements.

II.

It is known from the work of BRAUNSTEIN that the following reactions occur most actively in the presence of muscle tissue:

(1) oxaloacetic acid + l(+) glutamic acid ⇌ l(—) aspartic acid + α-ketoglutaric acid.
(2) pyruvic acid + l (+) glutamic acid ⇌ l (+) alanine + α-ketoglutaric acid.

There are considerable difficulties connected with the measurement of reaction rates in reaction (1), owing to the relative instability of oxaloacetic acid in muscle tissue. Its reduction to malic acid, decarboxylation to pyruvic acid and condensation to citric acid lead to unreliable results unless the complete balance sheet is drawn up for all of the substances concerned. There are no such difficulties in determining the reaction rate in reaction (2). We have chosen this reaction as the subject of our studies.

In principle we accept the nomenclature advanced by BRAUNSTEIN and call the enzyme aminopherase. As the aminopherase in question catalyzes only reaction (2), we think it advisable to call this enzyme glutamino-pyruvic aminopherase.

With the method, described below, we were able to purify glutamino-pyruvic aminopherase 550 times as compared with the original activity of the heart muscle tissue. The most active preparations are colorless. Different preparations obtained by somewhat altered procedures yielded the same maximal activity. Therefore, as attempts to purify the enzyme any farther have all failed, we believe that such preparations are practically pure.

We had no indication that the enzyme would require a coenzyme. The slight yellow colour invariably present in our preparations, shows no specific absorption bands and we do

not think its having anything to do with the enzymic activity.

Glutamino-pyruvic aminopherase brings about an equilibrium in reaction (2), there is less pyruvic acid than alanine in equilibrium.

We have determined two of the four enzyme-substrate dissociation constants, which are necessary to describe the reaction. The enzyme-alanine dissociation constant was found to be $1{,}5 \cdot 10^{-2}$ the enzyme-α-ketoglutaric acid dissociation constant: $1{,}7 \cdot 10^{-3}$. In spite of great deal of experimental work, we could not determine the similar values of the enzyme-glutamic acid and enzyme-pyruvic acid dissociation constants. This was mainly due to the lack of reliable and exact methods for the determination of other substrates than pyruvic acid.

III.

Methods.

Throughout this work the enzyme activity was determined by the amount of pyruvic acid formed or disappeared. STRAUB's salicilic aldehyde method[5] was used and we found it very satisfactory for the present purpose. None of the other three components gave any reaction with salicylic aldehyde, thus ensuring complete specificity. When muscle tissue, or a still impure enzyme was used, the protein was removed with the tungstate-sulfuric acid mixture (see STRAUB[5]). But at a rather low degree of purity, this deproteinization became superfluous as the amount of protein present in the test was negligible. In these cases 1 ml of the reaction mixture was pipetted into 1 ml of the concentrated KOH solution and 0,5 ml of the salicylic aldehyde reagent added. The protein originally present, was dissolved in the strong potash solution, without giving any opalescence. Nor did it give any colour with salicylic aldehyde.

We found that the method would give reliable results within 3% of error, if the following procedure was rigorously adhered to: The pyruvic acid solution (1 ml) was pipetted into a test tube, containing already 1 ml of the potash solution. 0,5 ml salicylic aldehyde solution was immediately added and the mixture thoroughly shaken up. Within a minute the test

tube was placed into a water bath of 38°, kept there exactly for 10 min., placed in an icebath for 10 minutes and the result read after this time. Determinations on pure pyruvic acid solutions have shown that in this case the extinction values (log I_0—log I) are directly proportional to the pyruvic acid concentration. The linear relationship will however break down if more than 0,3 mg pyruvic acid is taken for the determination. Should there be more pyruvic acid in solution, it should be correspondingly diluted. 0,1—0,3 mg pyruvic acid per ml is the ideal concentration to be taken for a determination. The readings were made with a Pulfrich photometer, using a cuvette of 1 mm thickness and the blue filter S47.

The following preparations were used as substrates: 1. l(+) glutamic acid. Its optical rotation was controlled in the presence of ten mols of HCl and was found to agree with the described value of $\alpha\,^{20}_{D} = +31°$. 2. Pyruvic acid was redistilled from the commercial product and a solution made up by acidimetric titration. 3. Alanine was used in the d, l form. As the d (—) form does not react at all, the concentration of l (+) alanine was taken only into consideration. 4. α-ketoglutaric acid was prepared synthetically from oxalosuccinic ester by the method of GABRIEL[6]. Its melting point was close to the theoretical value (111° instead of 112—113°). These preparations contained some impurity, which gave a colour with the salicylic aldehyde reagent. In the preparation mostly used in these studies, 1 mol/liter ketoglutaric acid gave a colour corresponding to 0,014 mol/liter pyruvic acid. The impurity, however, was not pyruvic acid, and corrections were taken for this value.

IV.

Test for the enzyme activity.

We have defined, as the glutamino-pyruvic aminopherase u n i t, the amount of enzyme, which formed 1 mg of pyruvic acid in 4 ml of the test solution within 15 minutes at pH 7,3 and 38°, when the initial concentration of l-alanine and α-ketoglutaric acid was 0,017 mol/liter each, the initial concentration of pyruvic acid and glutamic acid nil.

From 3—4 determinations with different quantities of an

unknown enzyme solution, this value is obtained by graphic interpolation. Fig. 1. shows such a determination. It is seen that the curve obtained is far from being linear. This is due to the fact that equilibrium is reached under these conditions if 2 mg pyruvic acid are formed.

The test solution was made up in the following way: Phosphate buffer of pH 7,3 to give a final concentration of 0,1 M, the enzyme solution and 0,5 ml of a 0,137 M l-alanine solution were placed in a test tube and filled up to 3.5 ml with dis-

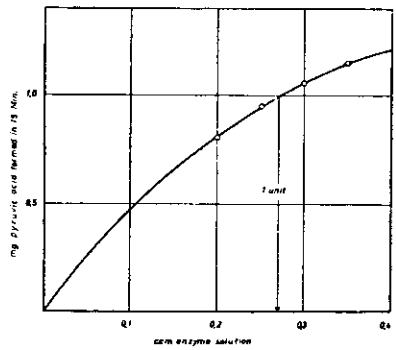


Fig. 1.

tilled water. The tube was then placed in a waterbath of 38°. The α-ketoglutaric acid solution (neutralized with NaOH) was placed in a test tube in the same waterbath and after 5 minutes, 0,5 ml of it was given to the test, mixed thoroughly and then left to stand in the waterbath for 15 minutes. It was found unnecessary to employ any shaking during the incubation. After 15 minutes the solution was either deproteinized by letting the required amount of sulfuric acid run into the tube, while still in the waterbath, or 1 ml of it were taken out and given immediately into 1 ml of potash solution. If there was too much pyruvic acid present, 1 ml of the test solution was taken out, mixed with 1 ml potash solution as before, and then diluted with a twice diluted potash solution to reach a convenient pyruvic acid concentration. From this dilution 2 ml were pipetted into a test tube and mixed with 0,5 ml salicylic aldehyde for the determination.

V.

Purification of the enzyme.

Pig's hearts were minced with an ordinary meat mincer. 800 g of the mince were mixed with 800 ml of a 0,1 M acetate buffer of pH 3,8 and left to stand at room temperature for 24 hours. The mixture was then warmed up to 60° and kept at this temperature for 15 minutes. Then the fluid was squeezed through a thin cloth. If neutralized to pH 7 by the addition of a 10% NaOH solution, a precipitate was formed. This is centrifuged off, leaving a clear, pink solution (A).

To 1080 ml of solution (A) thus obtained, 242 g ammonium sulfate were added and the precipitate, which contained the enzyme, was filtered off through fluted filters. (Ammonium sulfate: 0,375 saturation.) The filter paper, together with the precipitate was suspended in 80 ml of distilled water. The enzyme solution was sucked off from the filter paper pulp and the residue was washed with 20 ml of distilled water. The solution and washing were combined and dialyzed against distilled water at 0° for 24 hours. After dialysis we obtained 149 ml of a clear brown solution (B).

130 ml of solution B were acidified by the addition of 6,5 ml of a 2 M acetate buffer of pH 4,6 and then mixed with 12 ml of an alumina Cγ suspension (280 mg Al_2O_3). The enzyme was adsorbed on the alumina gel and separated from the solution in the centrifuge. The precipitate was stirred up with 20 ml of a 0,1 M phosphate buffer of pH 7,3 and the solution was adjusted to pH 6,8 by the addition of a few drops of a dilute NaOH solution. The precipitate was eluted for a second time by adding 20 ml of a 0,1 M phosphate buffer of pH 6,8 to the gel. After thorough mixing it was centrifuged off again and this elution was combined with the first one. We obtained in this step 39 ml of enzyme solution (C). It is advisable to make a preliminary test in order to find the optimal amount of alumina gel, i. e. the amount of alumina necessary to bring down about 60—70% of the enzyme.

33 ml of the solution (C) obtained in the previous step, were refractionated with ammonium sulfate between 0,30 and 0,375 saturation values. Both precipitates settled well in the centrifuge. The fraction, which precipitated between 0,3 and

0,375 saturation, was dissolved in distilled water, giving 6 ml of a pale yellow solution. It contained the enzyme with maximal activity. (D).

The purified enzyme keeps well in solution, especially well in an ammonium sulfate solution. In some cases we found no loss of activity during a period of two months, if the enzyme was kept in a 0.15 saturated ammonium sulfate solution in the icechest.

In Table I. the numerical details of the purification procedure are summarized:

Table I.

Step	Enzyme units/ml	Total units	Enzyme unit/mg
A	4,1	4450	1,03
B	16,5	2150	4,17
C	28,4	937	12,6
D	103	618	28,6

It is seen that from the first solution 14% of the activity are retained in the last fraction and the activity per dry weight is 28 times higher. Actually the most successful step of the purification is the first one, in which at the very acid pH and high temperature, most of the muscle proteins are denatured and removed. We find that 1 g of fresh muscle tissue contains 10 units of glutamino-pyruvic aminopherase, i. e. about 0,05 units per mg protein (dry weight). Therefore in the present case the first step means a 20-fold increase in activity and the whole procedure a 570-fold purification.

In different preparations, the final value of the purified enzyme lay always between 28—29 units/mg.

Properties of the enzyme. Glutamino-pyruvic aminopherase shows a remarkable stability towards heat and a considerable resistance towards acid pH. The first point was already observed by BRAUNSTEIN. If kept at room temperature for half an hour, the enzyme is inactivated by pH 3 and pH 11. It is stable between these pH values. Alcohol, but not acetone destroys the enzyme even at 0°. Dialysis against distilled water does not diminish the activity of the enzyme. We have tested at different stages of the purification the effect of boiled muscle juice and of boiled enzyme solutions on the activity of the enzyme. We could never find any rise in the activity.

It is consequently plain that the enzyme does not have a dissociable prosthetic group.

Part of the enzyme can be removed by extracting the muscle with distilled water or salt solution, part of it will, however, remain in the residue even after prolonged washing. It is the heat treatment with — or without — the acid ph which brings the enzyme into solution.

The specificity of the enzyme was not studied extensively. We found that if alanine is replaced by aspartic acid in the

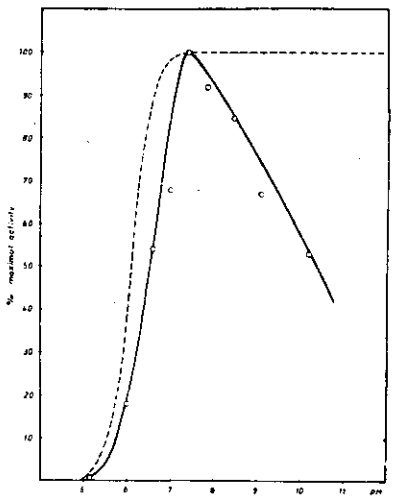


Fig. 2.

test, or aspartic acid and pyruvic acid are added to the enzyme, only traces of oxaloacetic acid are formed and no pyruvic acid disappears in the second case.

Fig. 2. shows the pH activity curve of the enzyme, when the reaction starts from alanine and ketoglutaric acid. The assimetry of the curve is an interesting feature. It is probable that on the acid side the activity is connected with the charge of the substrate, as shown by NORTHROP[7] for he pH-activity curves of pepsin and trypsin. The dotted line in Fig. 2. shows the percentage of negatively charged alanine ions as the function of pH. The enzyme seems to be able to react only with the negatively charged alanine ion and not with the positive ion or the zwitterion. It should be remembered, that the enzyme is not destroyed by even much lower pH values, than those at

which there is already no reaction. Preliminary experiments on the pH activity-curve of the enzyme for the reverse reaction show that this is shifted towards the alkaline side as compared with the curve of Fig. 2.

The experiments to determine the pH activity curve have been performed so as to get initial velocities. Therefore 2 units of enzyme were taken for the usual test solution of 4 ml. The reaction was started 5 minutes after the other ingredients were placed in the waterbath, by the addition of the ketoglutaric acid solution. It was stopped after 4 minutes by pipetting 1 ml of the solution into 1 ml of concentrated KOH solution. The buffers used were: glycine-HCl in the acid region, citrate-phosphate mixtures between pH 4,6—7,8 and glycine-NaOH on the alkaline side. All of them were present in 0,1 M final concentration. The pH values were controlled colorimetrically.

VI.

Kinetics of the reaction.

1. Equilibrium. The position of the equilibrium in the reversible reaction (2) was studied for the case of equal concentration of the two reacting substrates, under varyng enzyme concentration and varying initial concentration of the substrates. Table II. contains some of these results. It is seen that the equilibrium position does not depend on the concentration of the enzyme, nor on that of the substrate. From the mean value of 45,5% pyruvic acid in equilibrium, the equilibrium costant can be calculated:

$$K = \frac{C_{\text{alanine}} \cdot C_{\text{ketoglutaric acid}}}{C_{\text{glutamic acid}} \cdot C_{\text{pyruvic acid}}} = \frac{54,5^2}{45,5^2} = 1,43$$

2. Reaction velocity. The aim of kinetic investigations is the analysis of the mechanism of enzyme reactions. If the reactions, which take place during the enzyme action are set up correctly, we should be able to obtain an equation, which tells us the composition of the system at any given time. provided the initial concentrations of substrates and enzyme are given at a fixed pH and temperature.

There is no enzyme reaction for which such an equation

Table II.

Enzyme units/ml	Initial concentration mol/lit				pyruvic acid mol/lit found in equilibrium	%pvruvic acid in equilibrium
	l(+)glutamic acid	pruvic acid	l(+)alanine	α-ketoglutaric acid		
1,0	0,100	0,100	0	0	0,044	44,0
1,0	0,070	0,070	0	0	0,0309	44,1
1,0	0,040	0,040	0	0	0,0167	41,8
1,0	0,0200	0,0200	0	0	0,0094	47,0
1,0	0,0090	0,0090	0	0	0,00398	44,3
1,0	0,0050	0,0050	0	0	0,00241	48,2
1,0	0	0	0,0050	0,0050	0,00230	46,0
1,0	0,0025	0,0025	0	0	0,00122	48,8
1,0	0,0010	0,0010	0	0	0,00043	43
2,5	0,0060	0,0060	0	0	0,00278	46,4
0,82	0,0060	0,0060	0	0	0,00281	46,8
0,25	0,0060	0,0060	0	0	0,00276	46,0
					mean value:	45,5

is elaborated, owing no doubt to the complexity of the question. Several attempts were made earlier on kinetic studies of enzyme reactions, but most of them were connected with proving or disproving empirical laws. It was first realized by MICHAELIS and MENTEN[8] and later by WARBURG and his associates[9] that the mathematical solution of this question can be achieved by assuming that the enzyme reaction rate depends on the concentration of the enzyme-substrate complex. By determining the enzyme substrate dissociation constants they were able to obtain time-laws for some enzyme actions. But even from these studies no generalized time law could be constructed, owing no doubt to the aproximative nature of the calculations.

The necessity of work on these lines is emphasized by the fact that one often encounters kinetic studies in which the simple non catalytical time laws of monomolecular or bimolecular reactions are shown to hold for an enzyme reaction. COHEN for instance in his paper on transamination states that the transamination can be described by a bimolecular reaction constant. This can only be due to experimental error. Apart

from the fact that not even the reversibility of the system is taken into account, such an attempt is meaningless. This is illustrated by Fig. 3. in which the experimental time curve for the alanine-ketoglutaric acid reaction is indicated by the amount of pyruvic acid formed. Substrate concentration: 0,00685 mol/liter for each substrate, enzyme concentration 0,56 units/ml glutamino-pyruvic aminopherase. PH 7,3, temperature 38°. The time curves of a simple bimolecular reaction and that

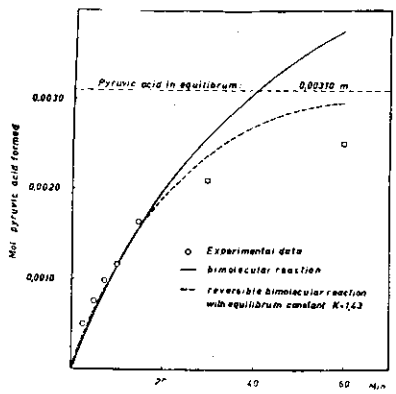


Fig. 3.

of a reversible bimolecular reaction (with equilibrium constant $K = 1{,}43$) are drawn into the same figure so as to meet the facts at one point. (10 min.). It is seen that they do not meet the facts at any other point.

If we assume that the substrates form dissociating complexes with the enzyme, it is possible to obtain an integral equation as the time law of this reversible bimolecular enzyme reaction. This is rather a complicated equation even for the case of equal initial substrate concentration, but it contains only the following quantities: initial concentration of substrates, enzyme concentration, the four enzyme-substrate dissociation constants, the equilibrium constant and the substrate concentration at time *t*. Owing to reasons described above, two of the enzyme-substrate dissociation constants are still missing and we were not able to prove our equation, thus not described here. But it is believed that only on these lines can the kinetic laws of catalyzed reactions be developed.

Determination of the enzyme-substrate

dissociation constants. We suppose that alanine and ketoglutaric acid, which react with one another are bound to different points of the enzyme. In this case the dissociation

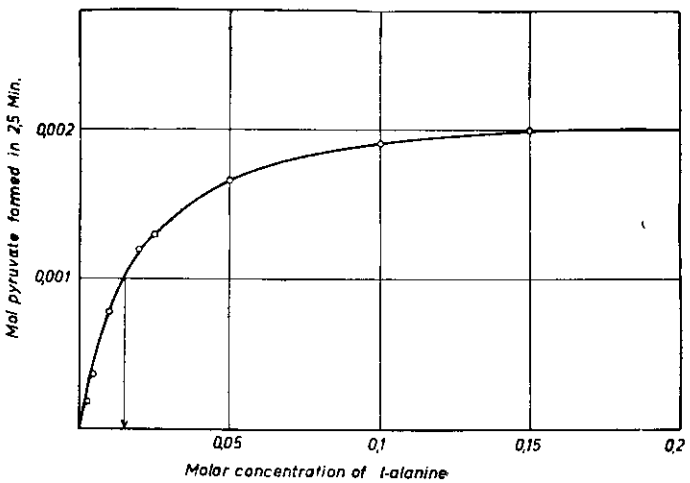


Fig. 4.

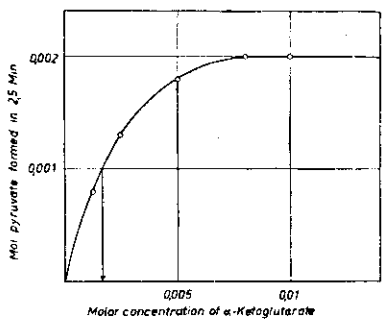


Fig. 5.

of the enzyme-alanine complex is independent of the concentration of the ketoglutaric acid and vice versa. If therefore the concentration of ketoglutaric acid is kept constant and so high that it saturates the enzyme (0,05 M), then the initial velocity is proportional to the concentration of the enzyme-alanine complex. If the initial velocities are now determined for varied alanine concentrations, the curve of Fig. 4. is obtained. The alanine concentration, at which half of the maximum velocity

is observed, is equal to the enzyme-alanine dissociation constant. In a similar way, the enzyme-ketoglutaric acid dissociation constant can be evaluated from Fig. 5. Here the concentration of alanine was kept constant and high (0,1 M) and the concentration of α-ketoglutaric acid varied.

We are indebted to Prof. V. BRUCKNER and Mr. C. AUTHERIED for a generous supply of synthetic α-ketoglutaric acid.

References :

1. *D. M. Needham,* Biochem. Journ. *24,* 208 (1930).
2. *I. Banga* and *A. Szent-Györgyi,* Z. physiol. Chemie *245,* 113 (1937).
3. *A. E. Braunstein* and *M. G. Kritzmann,* Enzymologia *2,* 129, 138 (1937), *ibid., 7,* 25 (1939), *M. G. Kritzmann, ibid., 5,* 44 (1938) and Biochimia *3,* 603 (1938).
4. *P. P. Cohen,* J. biol. Chem. *136,* 564, 585 (1940), *P. P. Cohen* and *G. L. Hekhuis, ibid., 140,* 711 (1941).
5. *F. B. Straub,* Z. physiol. Chemie *244,* 117 (1936).
6. *S. Gabriel,* Berichte d. deutsch. Chem. Ges. *42,* 655 (1909).
7. *J. H. Northrop,* Journ. gen. Physiol. *5, 263* (1922).
8. *L. Michaelis* and *M. L. Menten,* Biochem. Z. *49,* 333 (1913).
9. *O. Warburg* and *E. Negelein,* Biochem. Z. *282, 212* (1935).

CONTENTS