VI. THE SPREADING OF MYOSIN.

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WHEREAS most proteins so far studied show spreading at their isoelectric points or on strongly acid solutions (we have examined more or less thoroughly ovalbumin, zein, gliadin, insulin, pepsin, trypsin, ovoglobulin, ovomucoid, caseinogen, globin and haemoglobin) some proteins do not spread, when tested by our usual technique. Gelatin is a bad spreader and this is due to its too great solubility. Myosin and probably fibrinogen also do not spread because the particles of the sols have too little solubility.

Spreading of well-purified myosin is very difficult to obtain. At the isoelectric point (p_H 6.1) spreading is never observed, and even on a strongly acid solution p_H 1.0 sometimes no spreading can be detected.

In early experiments when the myosin was not sufficiently freed from myogen (or other contaminants) we obtained some spreading differing according to p_H . When the solutions were almost free from other proteins, we observed small films of protein floating on the surface, visible to the naked eye, and having a bluish tint. After more prolonged purification, this also was no longer observed.

Now it is possible to induce myosin solutions to spread by adding to the solution a trace of a proteolytic enzyme. Before degradation takes place into small particles which have no longer spreading properties (amino-acids never spread and neither do smaller peptides like the tetrapeptide of alanine and the tripeptide of leucine [Gorter *et al.*, 1934]) the myosin is transformed into a spreading substance; this process is highly dependent on the temperature of the solution and on the amount of the trypsin added, and it takes some time before the final effect of the action of the trypsin on the myosin is obtained, when the concentration of trypsin is rather low.

In Fig. 1 the results are given of measurements of the spreading of myosin solutions (6 mg./ml.) to which trypsin in one concentration 1/3250 was added. The temperature was kept constant (38°) and the results after various times were plotted.

It appears that before hydrolysis as indicated by the falling off of the curve occurs, the myosin is transformed into a group of spreading substances. Even during a certain length of time spreading remains maximum, although the enzymic process must be considered as being continuous.

Whereas the curve in Fig. 1 from 2 to 10 minutes appears linear, experiments with smaller amounts of trypsin indicate that this appearance is incorrect (Fig. 2).

Fig. 3 shows the influence of the concentration of trypsin on the spreading of myosin solutions of the same strength, time and temperature being constant (8 minutes and 38° temperature).

The next series of curves (Fig. 4) shows the results of measurements of spreading when different concentrations of trypsin are added to myosin (6 mg./ml.) after various periods of time at the same temperature. If the concentration is high (1/125), no maximum spreading is observed: the hydrolysis has proceeded too far before the first measurement can be made (2 minutes).

In the next series with a concentration of $1/350$, hydrolysis begins just after 2 minutes, when the maximum has been reached. At $1/1000$ the curve begins

Fig. 1. Influence of time of action of trypsin (final concentration $1/3250$) on spreading of myosin (6 mg./ml.). $T=38^\circ$. Tray solution $0.08 M$ phosphate.

— x — x — x — Myosin four times reprecipitated.
— + — + — + Myosin three times reprecipitated.

Fig. 2. Same as Fig. 1 save that trypsin concentration was $1/8000$. $T=40^\circ$. Differences between the curve are due to experimental error.

to fall after 4 minutes and at $1/2200$ both the beginning of the favourable influence on spreading and of the hydrolysis can be observed; the first process has already proceeded very far towards completion at the time of the first

measurement. The typical form of curve showing a gradual rise to a plateau followed by a fall appears at dilutions of 1/8000 and higher.

Fig. 3. Effect of varying concentration of trypsin on spreading of myosin. Time of action of trypsin 4 minutes. $T = 38^\circ$.

Fig. 4. Curves constructed similarly to those of Fig. 1 but with different concentrations of trypsin. Figures indicate relation between trypsin and myosin; e.g. 1/125 means 0.1 mg. trypsin and 12.5 mg. myosin in 2 ml. phosphate buffer.

In order to study the influence of temperature on the first part of the process, we have tried to get the same curve for different temperatures and different concentrations.

Fig. 5 shows that the same type of curve results when spreading is plotted

against time. From these observations a linear relationship between temperature and the logarithm of the trypsin concentration can be deduced (Fig. 6) from which a temperature coefficient of 3.6 for 10° can be calculated.

Fig. 5. Showing that the same area for the spreading of myosin after exposure for a given time to the action of trypsin can be obtained by varying both temperature and concentration of trypsin. If temperature is increased, the concentration must be lowered to get the same effect.

+—+—+ $T=16^\circ$,	and	trypsin/myosin = 1/350.
x—x—x $T=24^\circ$.	"	" = 1/1000.
•—•—• $T=40^\circ$.	"	" = 1/7900.
□—□—□ $T=48^\circ$.	"	" = 1/22,400.

Fig. 6. Showing linear relationship between the logarithm of the concentration of trypsin and the temperature. Temperature coefficient = 3.6. The point at 0° is obtained by extrapolation.

The maximum spreading per mg. myosin under these conditions is 0.9 sq.m. It is somewhat less than the maximum spreading of most other proteins at the isoelectric point.

All the above-mentioned measurements were made on 0.08 *M* phosphate buffer solution, having p_H 7.3–7.4. It is possible to show that the product obtained by adding trypsin to myosin at the moment when it begins to show maximum spreading is not very much influenced by p_H at about this reaction. We have obtained the same figure when examining "spreading myosin" in the same phosphate solution at p_H 7.1, 7.4, 7.6 and 8.1. This result was obtained by arresting the reaction by cooling the mixture to 0°.

We have also found that a muscle extract, probably containing the proteolytic enzyme from muscle, can in much smaller dilutions produce the same effect on a myosin solution as does trypsin.

The foregoing results seem interesting in so far as they show that an enzyme can, by inducing a protein to increase its surface, prepare it for hydrolysis into smaller peptides and amino-acids.

They have perhaps a still greater importance in other respects, because they show that there exist proteins which do not spread under the conditions that permit a spreading of most other proteins (at p_H 1.0, at the isoelectric point or after addition of bivalent negative ions on the acid side or of positive ions on the alkaline side of the isoelectric point), but that partial hydrolysis into simpler compounds is necessary to produce this spreading.

It is worth while to emphasise that this behaviour is observed with a protein, which shows birefringence of flow under polarised light, which is a proof of the existence of long-shaped particles in a myosin solution.

Preparation of myosin.

The myosin used in these experiments was prepared in a way similar to that indicated by Edsall [1930].

A rabbit was narcotised by means of a 20 % solution of urethane, injected intraperitoneally. The animal was bled to death by opening the carotid artery. The muscles of the legs and back were taken, weighed and quickly put through a mincer. The minced muscle was brought into about three times its weight of a concentrated salt solution containing 1.2 *M* KCl, 0.05 *M* Na_2HPO_4 and 0.01 *M* KH_2PO_4 . The mixture was kept at 0° and stirred vigorously for about 2 hours, during which time it became very viscous. It was strained through a linen cloth. The undissolved part was again mixed with salt solution (about a third of the quantity used the first time) and extracted at 0° during 2 hours. This was repeated until the solution no longer showed double refraction of flow and gave only a slight precipitate with salicylsulphonic acid. Usually three or four extractions sufficed.

The different solutions of muscle protein were combined and added slowly with careful stirring to twenty times their volume of ice-cold distilled water. The precipitate was centrifuged, re-dissolved in the smallest possible amount of the above-mentioned salt solution and the myosin reprecipitated by pouring into 20 vols. of ice-cold water. In this way the myosin was purified by three or four reprecipitations. The process of purification could be controlled by the spreading experiments: after some precipitations the product no longer spread, either immediately or after standing for an indefinite length of time.

The concentration of the solutions was estimated by means of micro-Kjeldahl determinations.

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