splitting are influenced by the CP–CPase system, when fibril bundles with a radius of *ca.* 1 μ are used as models. Inside such a thin fibril bundle (in contrast to the interior of fibre models) the ATP concentration is, to begin with, practically as high as in the bath. Consequently it cannot be increased further even by the action of the CP–CPase system.

3. These fibril bundles contract in exactly equal extent in the presence and absence of the CP–CPase system. This also holds good when the CP–CPase system is present in a concentration 300 times greater than that which exactly compensates the ATP splitting by the fibrils.

4. On the other hand, ATP contraction and splitting disappear immediately and completely when the "relaxing factor" of living muscles (discovered by MARSH) is added in the active state to the same fibril bundles.

5. Relaxing factor and CP–CPase system are thus not identical. Further, the relaxing factor is the only physiological agent known at present that is able to bring about relaxation and a state of rest in living muscle, as long as it is in the active state.

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THE INFLUENCE OF RELAXING FACTOR ON THE pH DEPENDENCE OF THE CONTRACTION OF MUSCLE MODELS

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I

In the experiments of BOZLER[1] the system phosphocreatine-phosphokinase (PC system) produces relaxation (through ATP restitution) by increasing the ATP concentration inside the muscle fiber model to the overoptimal concentration of the bath*** (see the preceding report of PORTZEHL).

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*** Physiological ATP concentrations are over-optimal if the models contain sufficient relaxing factor.

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Therefore, the effect of the PC system is indirect. These circumstances do not exist in living muscle.

In contrast to BOZLER, the school of SZENT-GYÖRGYI observed no relaxation with the PC system of muscle extract at pH 7 although they observed it at pH 6[2,3]. Thus the latter authors obtained a change from contraction to relaxation if the water-glycerin-extracted fiber bundle, in the presence of the PC system or muscle extract, was brought from pH 7 to pH 6. In the absence of both substances the amplitude between the tension at pH 7 and pH 6 was very small. How ATP-splitting was influenced under these conditions was not investigated.

II

In the experiments of the SZENT-GYÖRGYI school as well as in the investigations of BOZLER (see the preceding report of PORTZEHL) the relaxing factor of MARSH[4,5] may be involved; for in muscle extract and in thick fiber bundles it is always present if the bundles are not extracted for an extremely long time. Therefore, in the experiments reported in Fig. 1 only fiber bundles that had been extracted for two weeks or more, and consequently contained little or no factor, were used.

At pH 6.2 fiber bundles develop approximately 70% of the tension developed at pH 7.2. Further, it makes no difference if the highly active PC system used in the work of PORTZEHL was present (Fig. 1a, b). On the other hand, if a highly diluted extract containing relaxing factor is added, the fiber bundle contracts at pH 7.2 and almost completely relaxes at pH 6.2. (In higher concentration the relaxing factor also completely suppresses contraction at pH 7.2.) These findings are in so far in agreement with the results of LORAND, since the curve of LORAND showed a much smaller amplitude of tension between pH 7 and pH 6 in the presence of the PC system than in the presence of extract.

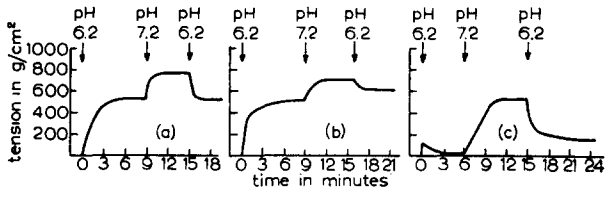

Fig. 1. The pH-dependence of tension in thick fiber bundles (diameter 200–300 μ). (a) in the absence of relaxing factors and PC system (average of 10 experiments); (b) in the absence of relaxing factor and presence of PC system (phosphocreatine, $10^{-2}M$ + PCase, average of 4 experiments); (c) in the presence of very dilute relaxing factors and in the absence of PC system (average of 4 experiments). ATP, $6 \cdot 10^{-3}M$; $MgCl_2$, $2 \cdot 10^{-3}M$; ionic strength 0.17 μ; relative length of fiber bundle 80% of standard length; room temperature.

Experiments with thick fiber bundles can very easily give false values because the ATP is used up in the outer layers of the fiber bundle. In consequence, (1) the largest portion of the contractile fibrils will not take part in the reaction, and (2) this portion will be in rigor. The thickness of the rigid core, however, must be different at pH 6 from that at pH 7, because the velocity of ATP splitting at pH 6 is only half as great as at pH 7. Consequently, the smaller tension of the single fibril at pH 6 is partially compensated by an increasing number of active fibrils produced by the transition from pH 7 to pH 6.

Fiber models of very thin single fibers (diameter of 50 μ to 60 μ) always adjust, at pH 6, to the correct equilibrium tension, irrespective of whether they are approached from above or below (Fig. 2a, 2b). This is the case even though such thin

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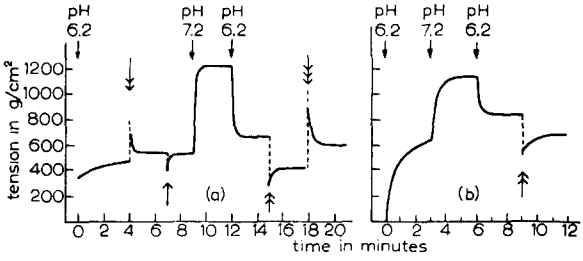

Fig. 2. Demonstration of equilibrium tension in thin single fibers (diameter approximately 72 μ) at pH 6.2. The equal tension in the first and second period at pH 6.2 and the weak swing of tension after "stretch" and "release" shows that the true equilibrium tension is reached. (a) without $CaCl_2$ (average of 3 experiments); (b) with $2 \cdot 10^{-3} M$ $CaCl_2$ (average of 3 experiments); ↓ stretch of 2.5 % of standard length = 7.5 % of the final length, ↑ release of 2.5 % of standard length = return to final length, ↕ release of 4 % of standard length = 12 % of final length, ⇕ stretch of 4 % of standard length = return to final length. ATP, $6 \cdot 10^{-3} M$; $MgCl_2$, $2 \cdot 10^{-3} M$; ionic strength 0.16 μ; relative strength, 33 % of standard length; room temperature.

single fibers at an ATP concentration of $6 \cdot 10^{-3} M$ already slightly exceed* the "Grenzschichtdicke".

If such thin single fibers are investigated in the same arrangement as previously used for fiber bundles, they show:

1. Tension is much higher because the inactive core is much smaller. The mean value of tension at pH 7, > 1000 g/cm², is about double the mean value of tension in bundles, even though the tension of single fibers was measured at a relative length of ~30%, while the measured tension of bundles in Fig. 1 was obtained at ~80% of standard length**.

2. In contrast to tension in the fiber bundle the tension of such single fibers at pH 6 is only about half as high as that at pH 7 (Fig. 3a).

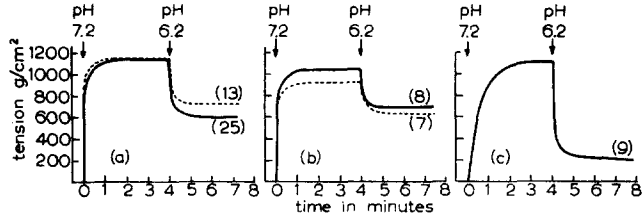

Fig. 3. Dependence of tension on pH in single fibers. (a) Fibers extracted (10–15 days), without relaxing factor and PC system. Mean values, the numbers associated with the curves indicate the number of experiments. Relative length, 33 %. ——— without $CaCl_2$ ------- with $CaCl_2$ $2 \cdot 10^{-3} M$. (b) the same in the presence of the PC system ($1 \cdot 10^{-2} M$ PC), relative length without $CaCl_2$ ~33 % ———, with $CaCl_2$ ~23 % -------. (c) The same in the presence of dilute "factor" solution, relative length ~33 %, ATP, $6 \cdot 10^{-3} M$, $MgCl_2$, $2 \cdot 10^{-3} M$; ionic strength, 0.16 μ.

3. The proportion of tension at pH 6 to tension at pH 7 is completely independent of whether $2 \cdot 10^{-3} M$ Ca^{++} is added or not. The single fibers are thus completely freed of relaxing factor through extraction from 14 to 30 days.

4. This proportion is also entirely independent of whether the PC-PCase system is absent or present in high concentration (Fig. 3b).

5. On the other hand, tension at pH 6—mean value of 9 experiments—is only 1/6 the tension at pH 7 if the relaxing factor is added in a dilution that just does not quite lower the tension at pH 7 (Fig. 3c).

* Measurement of tension of a single fiber at a relative length of 80 % is impossible because the tension is so high that the fiber tears itself.

** The ATP-free core amounts to between 1/5 and 1/2 of the total cross section. Yet this core is certainly no longer in a condition of rigor, since it is flooded with the split product of the outer section of the fiber, ADP. At a concentration of ~$5 \cdot 10^{-3} M$ and sufficiently high Mg concentration, ADP is a plasticizer scarcely worse than ATP.

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When under the same circumstances, that is, at a relative fiber length of 25–30%, single fiber models are investigated that still contain remnants of relaxing factor, in consequence of short extraction, the tension of such fibers at pH 6 in the presence of Ca^{++} is again half as high as at pH 7, because Ca^{++} inactivates the remaining relaxing factor (Fig. 4a). In the absence of Ca^{++}, on the other hand, the tension decreased at pH 6 to $^1/_3$ the tension at pH 7 under the influence of the remnants of the factor (Fig. 4a).

If the PC system is added to briefly extracted fibers that contain factor, relaxation immediately occurs, *i.e.*, the PC system in the presence of factor produces relaxation by raising the "inner" ATP concentration to the "outer" ATP concentration. This equilibration of the "inner" and "outer" concentrations, however, requires time. Thus the fiber, at pH 7, and in presence of a remnant of factor and of the PC system, develops at first an appreciable tension. This is followed immediately by a fall in tension, even at pH 7, as soon as the ATP inside had reached a sufficient concentration. At pH 6 the tension fell even closer to zero. The tension, after the ATP concentration "inside" has, through the PC system, become similar to that "outside", is about ¼ the tension at pH 7 (Fig. 4b).

III

These results show that:

1. The difference in tension between pH 7 and pH 6 is not at all influenced by the PC system in the fiber model completely free of relaxing factor or in the fiber model in which the remnant of relaxing factor has been inactivated with Ca^{++}. Under such conditions, the tension at pH 6 is always approximately half that at pH 7.

2. If traces of relaxing factor are present, the tension at pH 6 is markedly lowered while the tension at pH 7 is not yet or only slightly decreased. The tension amplitude under such circumstances, that is, between pH 6 and pH 7, is enlarged although the PC system is not present.

3. If besides relaxing factor the PC system is added, additional changes in tension appear. These additional tension changes depend upon an increase in the relaxation effect of the factor, for through the PC system the ATP concentration inside becomes nearer to the relatively high ATP concentration outside. If only very little factor remains in the fiber, then the tension at pH 6 alone is decreased because the factor clearly (see IV) has a greater effect at pH 6. If the quantity of remaining factor is larger, the tension decreases at pH 7 as well as at pH 6 (Fig. 4b); still the decrease is more at pH 6. If the factor concentration is further increased the tension at pH 7 finally disappears completely. This is the situation in BOZLER's experiments (see the preceding report of PORTZEHL).

IV

These experiments can be confirmed and broadened in a completely independent manner. When the active tension dependence on ATP concentration ($2 \cdot 10^{-3} M$ Mg^{++}, 0.16 μ) is measured with single fibers extracted for 7 weeks (*i.e.* with factor-free fibers) the tension does not depend on the ATP concentration "outside" from $10^{-4} M$ to $10^{-2} M$, as long as the ATP concentration "inside" is similar to the concentration

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"outside". Such is the case for a fiber ~50 μ thick as soon as the ATP concentration "outside" is greater than $5 \cdot 10^{-3} M$ at pH 7 and greater than $1 \cdot 10^{-3} M$ at pH 6 (see Fig. 5). If the ATP concentration falls below these critical values the tension decreases (Fig. 5), because as a consequence of the reduced diffusion gradient the inner part of the cross-section is no longer reached by ATP. When the PC system, however, is added to such factor-free single fibers the tension at low ATP concentrations increases to values that in the absence of the PC system were reached only at high ATP concentrations. The reason for this is that ATP restitution makes it possible for dilute ATP solutions to penetrate into the middle of the fiber. In contrast, no relaxing effect of the PCase system is observed with factor-free fibers up to an ATP concentration of $10^{-2} M$. Thus the presence of the PC system permits the investigation of the relaxing effect even at very low ATP concentrations.

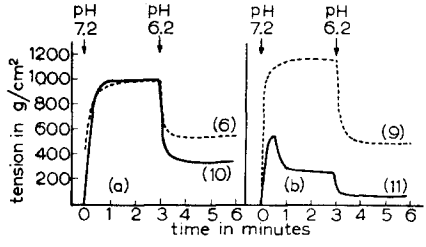

Fig. 4. Dependence of tension on pH in single fibers. The single fibers were extracted less than 4 days and contained traces of factor, (a) otherwise as in 3a, (b) otherwise as in 3b; ATP, $6 \cdot 10^{-3} M$; $MgCl_2$, $2 \cdot 10^{-3} M$; ionic strength 0.16 μ; relative length 27 %, room temperature, fiber diameter ~55 μ.

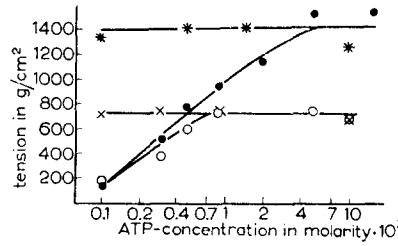

Fig. 5. Dependence of tension of single fibers on ATP concentration in absence and presence of PC system. Fibers extracted more than 7 weeks; curve 1: pH 7.2 without PC system (relative length ~33 %, average of 4 experiments) ●—●—●; curve 2: pH 6.2 without PC system (relative length ~33 %, average of 3 experiments) O—O—O; curve 3: pH 7.2 with PC system relative length ~20 % average of 4 experiments) *—*—*; curve 4: pH 6.2 with PC system (otherwise as in 3), ×—×—×. The total concentration of ATP plus PC equals $1 \cdot 10^{-2} M$. No Ca^{++}. Ionic strength, 0.16 μ; $MgCl_2$, $2 \cdot 10^{-3} M$; room temperature; fiber diameter 60 μ.

Such relaxing effects occur immediately if the relaxing factor is added, because the region of overoptimal ATP concentration is extended to ATP concentrations lower than $10^{-2} M$[6]. How far, in a single experiment, the overoptimal region of ATP concentration is decreased depends solely on the activity of the added factor, and not on the concentration of the PC system. The concentration of this system was the same in all experiments both at pH 6 and pH 7.

On the other hand, the activity of a given factor is much greater at pH 6 than at pH 7. Fig. 6 shows this for the activity used; at pH 7 an uninhibited contraction already takes place when the ATP concentration is decreased to $1 \rightarrow 3 \cdot 10^{-4} M$. In contrast, at pH 6 an uninhibited contraction occurs only when the ATP concentration is wholly an order of magnitude lower.

This section (IV) of the experiment shows the following:

1. Completely factor-free fiber model develops at pH 6 a tension half that at pH 7 so long as the "Grenzschichtdicke" is not exceeded significantly.

2. The "Grenzschichtdicke" of a single fiber is not exceeded at low ATP concentrations if the PC system is present in high concentration. Consequently the ratio 1:2 for the tension at pH 6 to the tension at pH 7 is maintained in factor-free fibers

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at low ATP concentrations, if PC system is present[3]. Other, and especially the relaxing, effects of the PC system fail at all ATP concentrations.

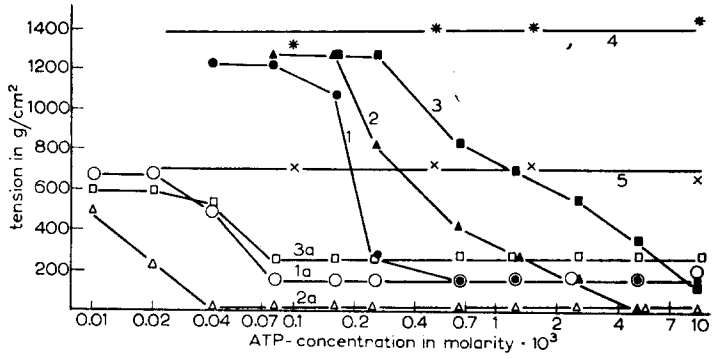

Fig. 6. Dependence of tension on ATP concentration in exhaustively extracted fibers (56–60 days) in presence of very dilute relaxing factor and concentrated PC system. Curve 1–3: at pH 7.2; curve 1a–3a: at pH 6.2 (relative length ~ 25 %); curve 4: at pH 7.2; curve 5 at pH 6.2 in presence of PC system alone (relative length ~ 20 %). The total concentration of ATP plus PC was $1 \cdot 10^{-2} M$. Ionic strength, 0.16 μ; $MgCl_2$, $2 \cdot 10^{-3} M$, without Ca^{++}, room temperature; fiber diameter ~ 60 μ.

3. The active tension at pH 6 decreases only to values less than half that at pH 7 if relaxing factor and ATP are present in concentrations that produce greater inhibition at pH 6 than at pH 7. For the relaxing factor concentration used in the experiments of Fig. 6, the corresponding ATP concentrations lie between $5 \cdot 10^{-4}$ and $5 \cdot 10^{-3} M$ ATP (Fig. 6).

4. The PC system does not influence the tension amplitude between pH 6 and pH 7 directly, but only insofar as it increases the ATP concentration inside the fiber.

5. Both the tension development of the completely uninfluenced contractile system as well as the inhibiting effect of relaxing factor are pH-dependent. Tension development at pH 6 is fundamentally smaller. The effect of relaxation factor at pH 6, as measured by the broadening of the region of overoptimal ATP concentrations, is much greater than at pH 7.

V

To sum up:

The difference between the experimental arrangements of BOZLER and of the SZENT-GYÖRGYI team is only that the content of relaxing factor in the experiments of the SZENT-GYÖRGYI school is much lower than that in BOZLER's experiments. Consequently the raising of the ATP concentration "inside" produces relaxation in BOZLER's preparations at both pH 6 and pH 7, while the comparable increase of ATP concentration "inside" the preparation of the SZENT-GYÖRGYI school produces relaxation only at pH 6, because the effect of relaxing factor is very much greater at pH 6 than at pH 7.

Since the relaxing factor is only effective if the ATP concentration "inside" exceeds a definite minimal amount, one may say in advance that every enzyme system that raises the concentration of ATP inside a fiber model containing "factor" sufficiently high will have a relaxing effect. Such systems are all systems that restore

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the ATP split with sufficient velocity. Actually, LORAND[7] has found in the meantime that the phosphoenolpyruvate-system also executes such an indirect relaxing effect (*cf.* also GOODALL[7a]). In contrast to the effect of relaxing factor such indirect effects are unphysiological, because in *living* muscle the ATP concentration is so high that, as long as the factor is not inactivated, relaxing factor inhibits splitting and contraction.

METHODS

Standard length psoas fibers were obtained by killing a rabbit and storing it at 10° C for approximately 3 hours before removing the fiber bundles. These bundles were then extracted and stored at —18° C in pH 6.9 phosphate buffered 50 % (w/v) glycerol. On three successive days the fiber bundles were divided into progressively smaller bundles in order to achieve the best possible conditions for extraction. The extraction medium was changed for three consecutive days and thereafter only irregularly over the period on extraction.

Tension was measured as described by A. WEBER[8].

Relaxing factor was prepared according to PORTZEHL (see the preceding report of PORTZEHL).

Adenosinetriphosphate creatine transphosphorylase was prepared by method B of KUBY, NODA AND LARDY[9] up to the crystallization step. Crystallization was not carried out.

Adenosinetriphosphate was obtained from Henning and from Pabst.

Phosphocreatine was supplied by the Sigma Co.

ACKNOWLEDGEMENT

The authors wish to acknowledge the very important contributions made by Dr. H. H. WEBER to these experiments through many helpful discussions.

SUMMARY

1. The true ratio of tensions of glycerol-extracted muscle fibers at pH 6 to pH 7 is 1 : 2.

2. This ratio is uninfluenced by the presence of creatine phosphorylase and phosphocreatine.

3. When the true ratio is obscured by employing muscle fibers of excessive diameter or fiber bundles or by utilizing ATP concentrations too low to penetrate the muscle fiber, then the PC system by restoring ATP will reveal the true tension ratio.

4. Although relaxing factor is effective at both pH 6 and pH 7 it is effective at pH 6 over a wider range of concentrations of ATP than at pH 7.

5. Thus the "relaxation" produced by the PC system depends either on the presence of relaxing factor or on revealing the true equilibrium tension when this is obscured by inappropriate experimental techniques.

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