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HYDROGEN-ION CONCENTRATION CHANGES IN FROG'S MUSCLE FOLLOWING ACTIVITY

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The *p*H changes in stimulated muscle have previously been followed by three methods:

(1) By measuring carbon dioxide gas exchange.

(2) By the glass electrode.

(3) By indicators [Margaria & Pulcher, 1934].

The attributes of the first two methods will be discussed briefly.

(1) *Carbon dioxide exchange.* The method is due to Lipmann & Meyerhof [1930]. The muscle was stimulated in fluid at room temperature and carbon dioxide exchange between the muscle and gas space was necessarily slow. The stimulation was in short bursts at regular intervals and the readings were taken at half-hour stages. There was no possibility of investigating the kinetics of the changes following a single short tetanus. It was not possible to study the changes with oxygen present probably for the following reasons: (1) there is complication due to usage of oxygen and production of carbon dioxide; (2) the reactions occur so quickly at room temperature that oxidative recovery would be sufficiently rapid to keep the muscle in a "steady state" with the rate of stimulation employed. No overall changes would be observed and the time lag is too great for transient ones to be measured. With a muscle in oxygen it is possible to stimulate it so much that the total catabolism shall progressively increase; but finally, at room temperature and with a muscle as thick as a frog's gastrocnemius or sartorius the rate of diffusion of oxygen would be insufficient to supply the needs of the innermost layers.

(2) *The glass electrode.* The method employed by Dubuisson [1937, 1939] has the advantages of being specific to *p*H changes and of rapidity in recording. The only time lag is caused by transmission of changes across the layer of fluid immediately surrounding the muscle. It is not possible to follow *p*H changes during oxidative recovery.

The method to be described here depends upon carbon dioxide exchange but differs essentially from that of Lipmann & Meyerhof [1930] because the muscle is supported in the gas space. This so reduces the time lag as to permit investigations of the kinetics of the changes following a single tetanus and of the changes occurring with oxygen present. Diffusion in the muscle is allowed for by calculation: errors so introduced are rendered unimportant by lowering the temperature to 0° C. The rate of metabolism is thus reduced much more than the rate of diffusion. Events during the contraction are much too transient to be studied by this means. For measuring short period changes (lasting a few seconds) the glass electrode method is clearly superior, but for recording *p*H changes which accompany some recovery events in frog's muscle at 0° C. the method to be described here is preferable owing to the stability of the base line over periods of 60 min. or more. For some of the slow changes which are investigated there is practically no correction for diffusion.

Method

Two identical frog's sartorii are fitted into the bulbs of a differential volumeter. The bulbs are filled with a mixture containing carbon dioxide. Either muscle can be stimulated and the resulting gas exchange is registered by observing the movements of the index drop in the capillary. All details of the apparatus and method of recording are given elsewhere [Hill, 1940*a*]. The volumeter was kept at 0° C. except where otherwise stated.

*Control of p*H. *Factors governing the rate of exchange of* CO_2 *following a change in p*H. For the following reasons it is important that the initial *p*H of the muscles be known. (1) The *p*H change due to hydrolysis of phosphocreatine is dependent upon the initial *p*H [Meyerhof & Lohmann, 1928]. (2) Lactic acid production is inhibited by a low *p*H [Kerly & Ronzoni, 1933].

The solution used for the preliminary soaking is brought to the required *p*H with bicarbonate-CO_2 buffer. The CO_2 mixture used for bubbling through the solution is the same as is used for filling the chambers of the volumeter. The range of *p*H required is 5–9. The concentrations of bicarbonate and CO_2 required are calculated from the following equilibrium relations:

$$\frac{[CO_2]}{[H_2CO_3]} = 700 \qquad \frac{[H^+][HCO_3^-]}{[H_2CO_3]} = 2 \times 10^{-4}.$$

The solubility coefficient of $CO_2 = 1$.

An isotonic solution of sodium bicarbonate is prepared and the calculated quantity added to Ringer's solution. Not more than 40% of the soaking mixture must be isotonic bicarbonate or the muscle develops spontaneous twitching: thus to work at *p*H 10 it is necessary to use only 0·04% CO_2.

At a given *p*H it is desirable, for the following reasons, to buffer with high concentrations both of bicarbonate and CO_2. (1) With a very small percentage of CO_2 there is a danger of the supply of CO_2 in the chamber being exhausted. A muscle at *p*H 6 with oxygen uptake inhibited develops irreversible alkaline changes on stimulation, and about 1 mm.^{3} CO_2 is absorbed following a 6 sec. tetanus. The volume of each bulb of the volumeter is 5 c.c. If five runs are taken the gas mixture must not contain less than about 0·1% CO_2. (2) When an equivalent X of acid is suddenly liberated in the muscle fX of H_2CO_3 is formed immediately. The rate at which CO_2 is liberated will be proportional initially to fX. The fraction f is less than, but approaches, unity as the efficiency of buffering increases. A similar argument applies to an alkaline change. It can be shown that in the range of buffer mixtures that are practically available at *p*H 6 the value of f may rise from 0·4 (for 1% CO_2) to 0·98 (for 50% CO_2). In conjunction with this it is important to consider the rate of the reaction $H_2CO_3 \rightleftarrows H_2O + CO_2$. The concentration of carbonic anhydrase in frog's muscle is 2–3% of that in blood and the reaction in either direction is therefore 90% complete in a few seconds. Provided the buffering is sufficiently good, there will only be a few seconds' delay in translation of a *p*H change into an equivalent change in CO_2 concentration.

Results

(1) *p*H 9 with normal oxygen consumption.

(2) *p*H 6–7·5 with normal oxygen consumption.

(3) *p*H 6 with oxygen consumption completely inhibited by cyanide.

(4) *p*H 6·5–8·5 with oxygen consumption completely inhibited by cyanide.

(5) *p*H 6·5–8·5 with oxygen consumption partially inhibited by sodium azide.

(1) *p*H 9 *with normal oxygen consumption*

At *p*H 9 there is practically no development of alkalinity accompanying breakdown of phosphocreatine. Following a tetanus of 6 sec. there is, however, a slow absorption of gas which is not complete for 20–30 min. (Fig. 1). The explanation is that the respiratory quotient of an active muscle is 0·9 [Gemmill, 1934] and therefore the volume of oxygen absorbed during recovery is greater than the volume of CO_2 produced. By finding the oxygen consumption under the same conditions it has been shown that the magnitude of the change observed is consistent with this value of the respiratory quotient.

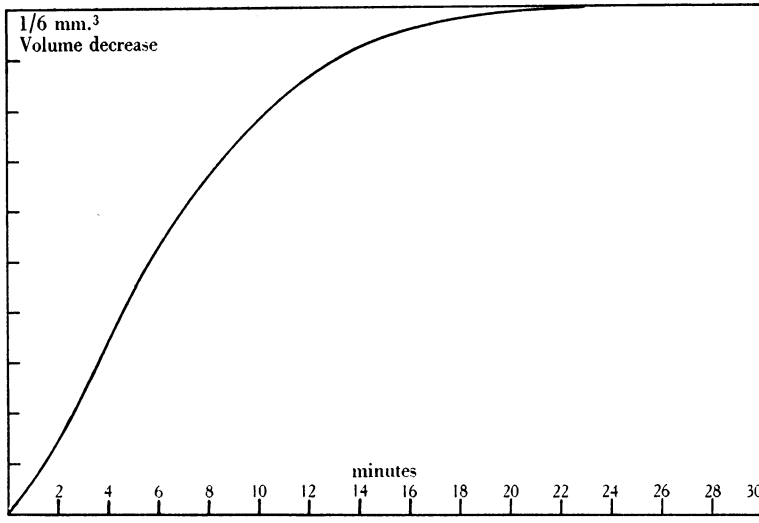

Fig. 1. Record of movement of index drop following 0·2 min. tetanus of muscle at *p*H 9 in an atmosphere of oxygen and CO_2. Oxygen consumption not inhibited. Muscle 80 mg. Total volume decrease $\frac{1}{6}$ mm.³

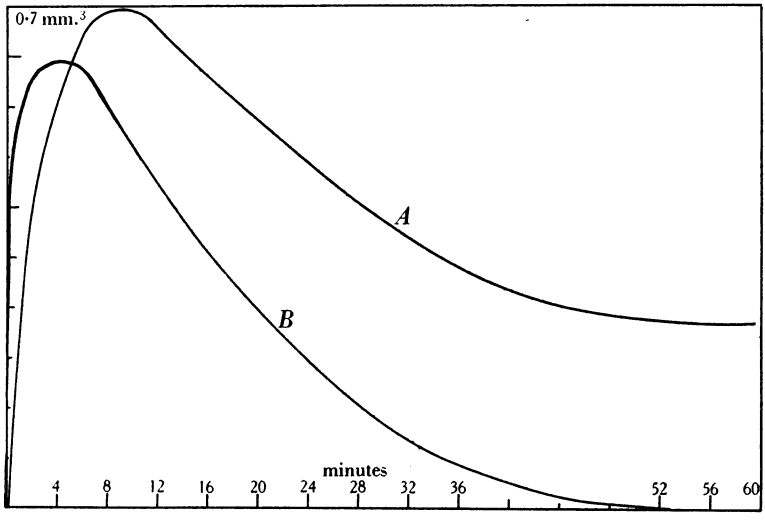

Fig. 2. A, record of movement of index drop following 0·2 min. tetanus at *p*H 6·5. B, after correction for oxygen consumption and CO_2 production (by subtraction of curve shown in Fig. 1) and for diffusion. Weight of muscle 100 mg.

(2) *p*H 6–7·5 *with normal oxygen consumption*

The curve followed by the index drop after a 3 sec. tetanus is shown in Fig. 2. The absolute height of the alkaline peak is dependent upon the initial *p*H since it is partly due to hydrolysis of phosphocreatine. The early rate of absorption is so rapid that the initial heat expansion is completely obscured. Resynthesis of phosphocreatine is slow and the index drop does not finally come to rest for 30–40 min. The final displacement of the base line is determined by the respiratory quotient.

To obtain the time course of change of *p*H the following corrections must be made: (1) For heat production of the muscle. (2) For diffusion of CO_2 in the muscle. (3) For the combined effects of utilization of oxygen and production of CO_2. The corrections (1) and (2) are made in a manner described in a previous paper [Hill, 1940*a*] and correction (3) is effected by subtracting the curve shown in Fig. 1 after scaling for equal final maxima. The results of this analysis is also shown in Fig. 2. The curve represents the balance between two processes, the breakdown and resynthesis of phosphocreatine. At the plateau the rates of the two processes are equal. The next experiment gives a better indication of the time course of breakdown since the complication due to resynthesis is avoided.

In the past the aerobic resynthesis of phosphocreatine has been followed by chemical methods, but no exact time course has been obtained. Neither volume change methods nor the glass electrode can be used on a muscle with adequate oxygen supply. Von Muralt [1934] measuring light transparency changes in muscle was able to follow phosphocreatine resynthesis in a muscle fully supplied with oxygen. He found that, at 20° C., the resynthesis following a 2 sec. tetanus is complete in 5 min. With a temperature coefficient of 2·5 for 10° C. this would be in agreement with the results obtained here.

(3) *p*H 6 *with oxygen consumption completely inhibited*

Kerly & Ronzoni [1933] have shown that lactic acid production in minced muscle is inhibited by acidifying the medium to *p*H 6. It will be seen that this is true also for intact stimulated muscle which has been soaked in a solution buffered at *p*H 6. Inhibition of lactic acid production by this means is preferable to the use of iodoacetic acid because the objectionable effects associated with the latter's action are avoided. A muscle can be kept at *p*H 6 indefinitely without damage provided oxygen is present: resynthesis of phosphocreatine occurs normally as shown in (2).

The oxygen uptake is inhibited by soaking for 1 hr. in Ringer's solution containing $M/1000$ sodium cyanide. Fig. 3 shows the result of a 3 sec. tetanus. It is seen that there is neither production of lactic acid nor resynthesis of phosphocreatine. The analysis in this case simply consists in correction for diffusion and for the initial heat production. The diffusion constant used here and in (2) is derived from the value found by Wright [1934] for frog's muscle at 22° C. by allowing a 1% decrease per 1° C. fall in temperature. In Krogh's units the diffusion

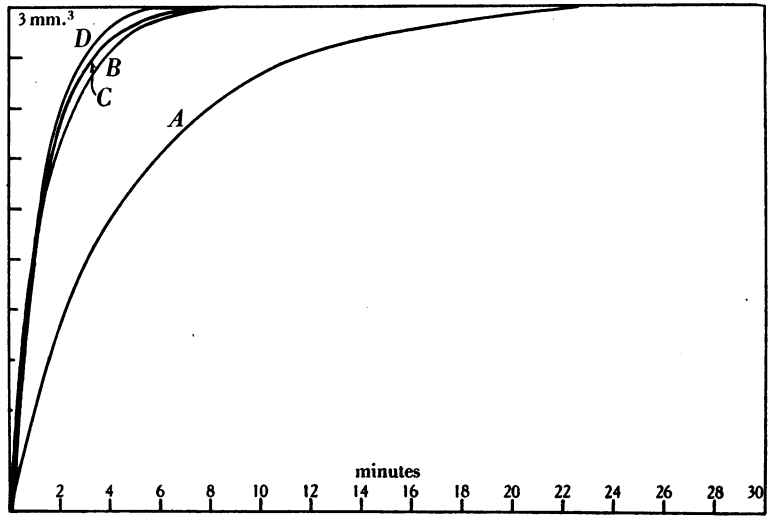

Fig. 3. *A*, absorption of CO_2 following 0·2 min. tetanus at *p*H 6. Temperature 0° C. Oxygen uptake inhibited by cyanide. Thickness of muscle 0·9 mm. *D*, absorption of CO_2 following ½ sec. tetanus at 16·3° C. *C*, the same but with times multiplied by the ratio of diffusion constants at 16·3° C. and 0° C. *B*, calculated control curve for thickness 0·9 mm.

constant at 0° C. is $5{\cdot}3 \times 10^{-4}$. The calculated curve for instantaneous disappearance of CO_2 is also shown in Fig. 3. It has not been found possible to obtain this control curve experimentally. The nearest approach that can be made is by stimulating the muscle under the same conditions but at a much higher temperature. The curve resulting from a ½ sec. tetanus at 16·3° C. is also shown in Fig. 3. It was with the same muscle as had been used at 0° C. For comparison with the calculated curve the abscissae are multiplied by the ratio of the diffusion constants at 16·3 and 0° C. The result shows that there will be no gross error from using the calculated curve for the analysis.

The curve corrected for diffusion is shown in Fig. 4. There appears to be a *sudden* alkaline change during activity followed by a slower change which is not complete for more than 10 min. It is probable that the curve gives the time course of phosphocreatine breakdown. Lundsgaard [1934] has shown that a certain fraction of phosphocreatine breakdown occurs *after* the relaxation, and the alkalinity phase which Dubuisson [1939] attributes to the same cause occurs *after* the relaxation.

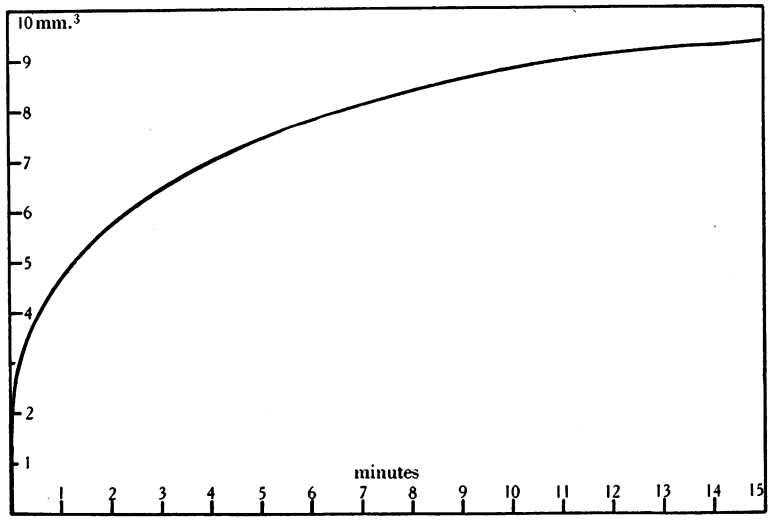

Fig. 4. Time course of the alkaline change following 0·2 min. tetanus. The conditions are such that oxygen consumption and lactic acid production are inhibited.

There is a marked resemblance between the analysed curve of Fig. 4 and the curve of anaerobic delayed heat production for a late member of a series under "alactacid" conditions [Hill, 1940*b*]. Both curves are half complete in about 1 min. It was suggested that the early stages of the anaerobic delayed heat accompanied the breakdown of phosphocreatine.

(4) *p*H 6·5–8·5 *with oxygen consumption completely inhibited by cyanide*

In this range of *p*H lactic acid production is not inhibited. To lessen, but not to abolish, the effect due to breakdown of phosphocreatine a *p*H of 7 is chosen and the effect of stimulation is shown in Figs. 5 and 6. There is first a heat expansion (at this *p*H it is not obscured by the concomitant alkaline change): next an absorption of CO_2 which starts rapidly but soon slows up and then reverses. The curve recrosses the

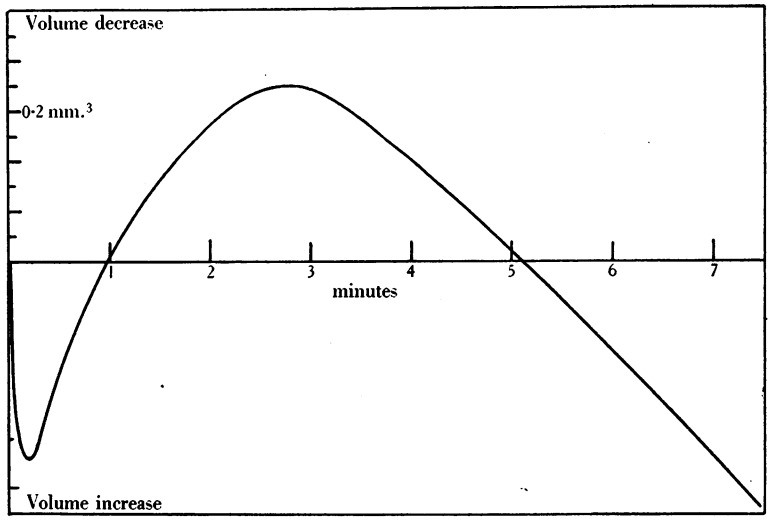

Fig. 5. Early stages of CO_2 exchange following 0·2 min. tetanus of muscle at *p*H 7 with oxygen consumption completely inhibited by cyanide.

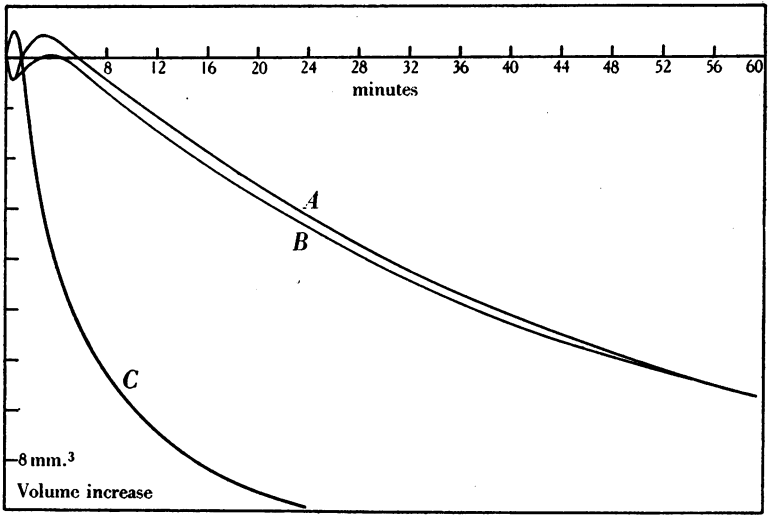

Fig. 6. CO_2 exchange following tetanus of muscle with oxygen uptake completely inhibited by cyanide. *A*, complete record at *p*H 7; the early stages are shown on a larger scale in Fig. 5. *B*, complete record at *p*H 8·5. *A* and *B* are both at 0° C. with 0·2 min. tetanus. *C*, at *p*H 7·5, 17° C. following a 1 sec. tetanus.

base line at about 5 min. and the output of CO_2 then continues for more than 1 hr. At *p*H 8·5 there is no change of *p*H due to breakdown of phosphocreatine: the initial heat expansion in that case is followed by a return toward the base line as the heat is dissipated, but the acidity reversal supervenes before the base line is crossed.

There can be little doubt that the prolonged acidity expansion is due to lactic acid formation. It is impossible to say exactly where this phase commences owing to the complication, in the early stages, due to the heat expansion and phosphocreatine breakdown. There would be no advantage in correcting the curve for diffusion, since only the early part where rapid changes are occurring would be materially affected and it is this region which has doubtful significance owing to summation of coincident changes.

The same experiment at 17° C. The form of the curve is the same as at 0° C. but with the time axis diminished (Fig. 6). For the same total lactic acid production the rate of formation at any time is about 5 times greater at the higher temperature.

The time course of lactic acid production has not previously been studied at 0° C. At room temperature the subject has been tackled by various methods: (1) chemical analysis [Lehnartz, 1931; Meyerhof, 1931*a*, *b*, *c*; Lundsgaard, 1931]; (2) volume change [Meyerhof, 1934]; (3) light transmission [Von Muralt, 1934]; (4) glass electrode [Dubuisson, 1937, 1939; Dubuisson & Schulz, 1938]. None of these methods, however, has been capable of determining the time course accurately. Dubuisson, for example, has stated nothing more explicit than that the acidity change is complete "within a few seconds" after contraction.

(5) *p*H 6·5–8·5 *with oxygen consumption partially inhibited by azide*

Activity oxygen consumption can be inhibited to any extent desired by poisoning with the appropriate concentration of sodium azide [Hill, 1940*a*]. It was therefore anticipated that progressive application of azide to a muscle at *p*H 7 should result in a continuous change from the aerobic type of curve (Fig. 2) to the anaerobic type of curve (Fig. 6). This proved not to be the case. The results using 3 sec. tetani are shown in Fig. 7. The azide concentration was increased from *M*/50,000 to *M*/4000 (with *M*/4000 the activity oxygen consumption is practically abolished). With *M*/50,000 azide the aerobic type of curve was obtained showing a slow resynthesis of phosphocreatine. As the concentration of azide was increased the only change observed was a decrease in the rate of resynthesis. Even with *M*/4000 azide the lactic acid phase was

completely absent. The addition of cyanide restored the ability to form lactic acid.

Recovery oxidation can, therefore, be completely stopped and yet no lactic acid is formed when the muscle is stimulated for 3 sec. A possible explanation might be that resting oxygen consumption (which is not abolished by azide [Stannard, 1939] is sufficient to destroy lactic acid or its precursors as fast as they are formed. If this is the case an increase in the duration of stimulus should raise the rate of production

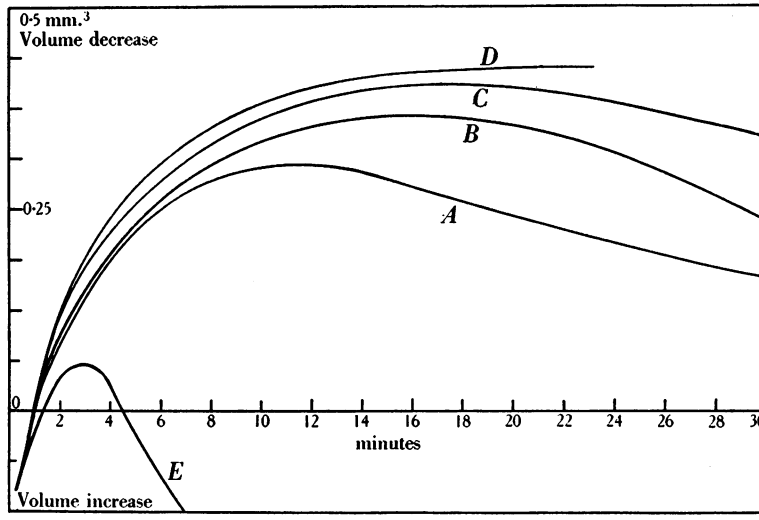

Fig. 7. Carbon dioxide exchange of muscles with oxygen consumption partly inhibited by azide. *p*H 7. 0·05 min. tetanus. A, *M*/50,000 azide; B, *M*/30,000 azide; C, *M*/15,000 azide; D, *M*/4000 azide. Compare E, oxygen consumption completely inhibited by *M*/1000 cyanide. Even with *M*/4000 azide there is no lactic acid production following a 0·05 min. tetanus.

of lactic acid (or its precursors) to a level at which the small resting oxygen consumption is incapable of maintaining a steady state. Lactic acid would then have to accumulate. A muscle, treated with *M*/4000 azide was stimulated for 3, 12 and 36 sec. with intermediate soaking at room temperature to hasten the later stages of recovery. The early stages of the resulting curves are shown in Fig. 8. After a 3 sec. tetanus there is no progressive lactic acid formation: with a 12 sec. tetanus the lactic acid phase appears, the curve crossing the base line at about 10 min.: with a 36 sec. tetanus the curve crosses the base line at 4 min. and is now similar to that obtained when oxygen consumption is completely

abolished by cyanide. In the last case the effect of the residual resting oxidation is negligible, the rate of production of lactic acid having been raised overwhelmingly.

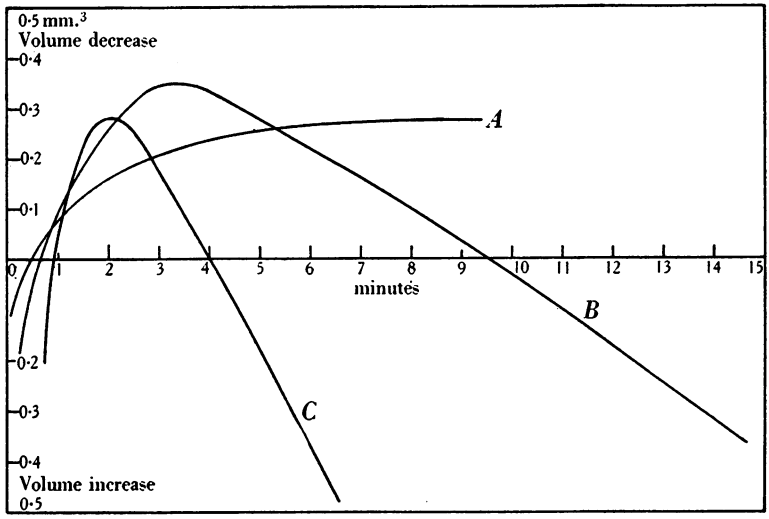

Fig. 8. Effect of changing duration of tetanus with muscle poisoned by $M/4000$ azide. A, 3 sec. tetanus; B, 12 sec. tetanus; C, 36 sec. tetanus. 0° C., pH 7.

Discussion

It has long been known that, in a muscle fully supplied with oxygen, there is no lactic acid formation following a short tetanus. This fact was frequently used as evidence for the theory that lactic acid is the substrate normally oxidized in a stimulated aerobic muscle. Other theories now hold sway but it is interesting to observe that the results of this paper militate against the older view. The argument is as follows. It has been seen in a previous paper [Hill, 1940*a*] that the rate of oxygen consumption following a short tetanus is at a maximum near the beginning of recovery and declines approximately exponentially. In other words, the activated substrate which is oxidized is formed rapidly during, or closely after, the contraction. On the other hand, lactic acid is formed extremely slowly. There is no possibility, therefore, that lactic acid is identical with the substrate normally oxidized in aerobic activity.

The conclusion must be that the formation of lactic acid occurs along a branch reaction which is independent of the main channel of oxidation. This is in harmony with present views on the chemistry of muscle.

It should not be inferred that lactic acid or its precursors cannot be oxidized. On the contrary, there is no reason to suppose that the reactions tending to the formation of lactic acid are not pressing forward even when oxidative recovery is permitted and the non-appearance of lactic acid must be due to oxidation of lactic acid or its precursors at the rate of formation. This subsidiary oxidation must be partly via cytochrome (for inhibition of cytochrome greatly facilitates the production of lactic acid) and partly via another respiratory enzyme refractory to the poisoning action of sodium azide. This latter system is capable of destroying lactic acid (or its precursors) at the rate at which it is formed following a 3 sec. tetanus at 0° C. but cannot restrain the accumulation of lactic acid after a longer tetanus. Comparison with the results of a previous paper [Hill, 1940*a*] shows that anaerobic recovery with lactic acid formation is considerably slower than aerobic recovery under the same conditions. The oxidation system concerned with prevention of lactic acid formation is thus presumably of minor importance. It should be considered as a mechanism for disposing of the end-products from non-oxidative recovery reactions. When oxygen is present these recovery processes are assisted by the more rapid ones which are primarily dependent on the progress of an oxidative reaction.

Summary

1. Changes in *p*H of stimulated frog's muscle are followed by the method of carbon dioxide exchange using a differential volumeter.

2. The muscle is supported in the gas space and it is possible to make the necessary corrections for diffusion and so obtain the time course of *p*H changes following a single short tetanus. Greater accuracy in this respect is attained by working at 0° C. and so slowing the metabolic processes. Changes of *p*H *during* the tetanus cannot be followed by this method.

3. When oxygen usage is permitted or in other circumstances when lactic acid is not formed (e.g. anaerobically at *p*H 6) alkaline changes only are observed. These accompany the breakdown and resynthesis of phosphocreatine and the time courses of these processes can be deduced.

4. At *p*H 7 with oxygen uptake inhibited by cyanide the production of lactic acid is not complete for more than 1 hr. It is half complete in about 15 min.

5. When the activity oxygen uptake is inhibited by sodium azide (the resting oxygen uptake being unaffected) the time course of formation

of lactic acid is dependent upon the duration of tetanus. If the latter is reduced below a critical value no lactic acid is formed. The significance of this is discussed.

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