17th European Meeting of Meat Research Workers

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Symposium 1

Mechanisms Controlling Glycolysis in Muscle

The Biochemistry of Post Mortem Glycolysis

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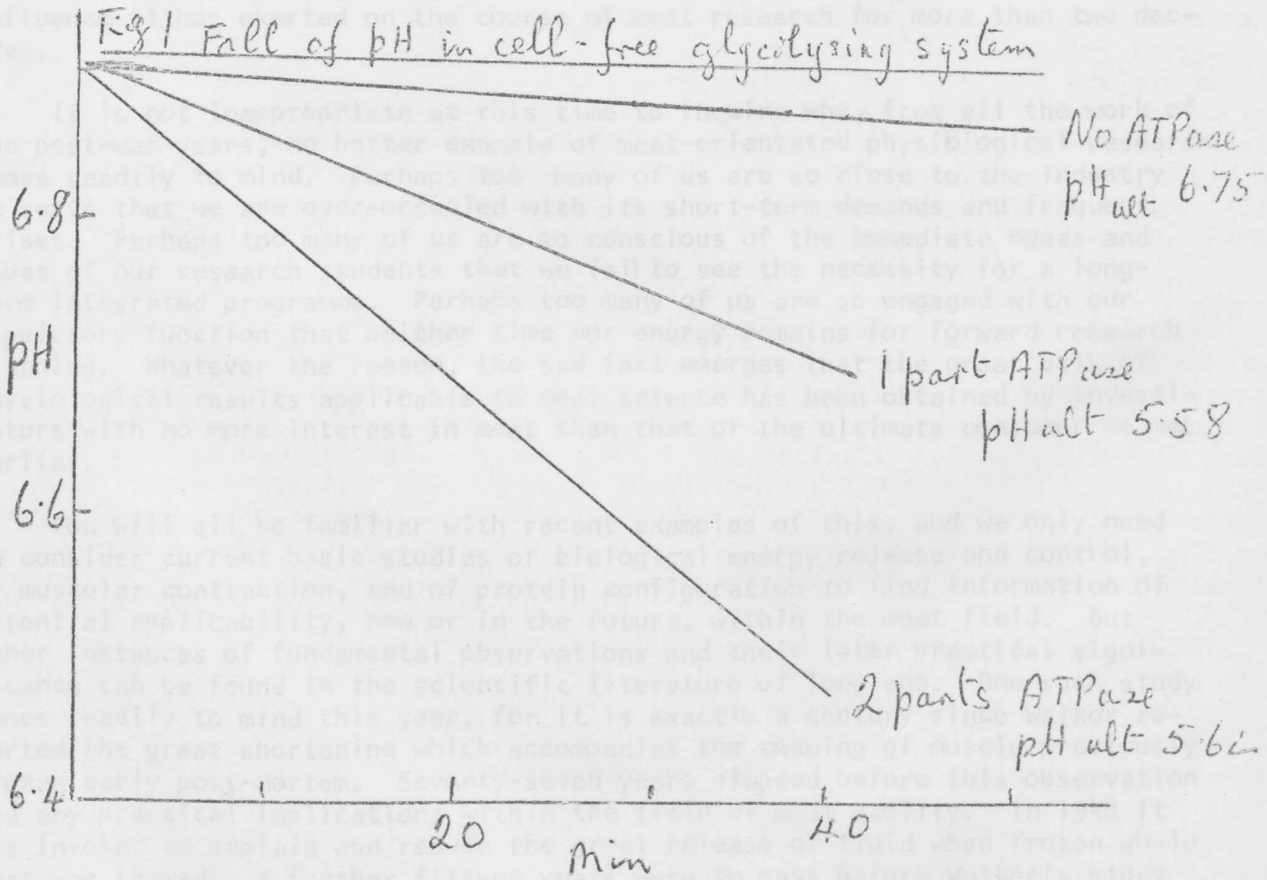
The most important changes that occur during the transition from living muscle to dead meat are associated with the anaerobic energy metabolism systems that exist in the muscle cells. These are firstly and most familiarly the glycolytic system which converts glycogen to lactate, and secondly those processes which lead to the dephosphorylation of ATP. Acidification occurs in parallel with glycolysis, rigor mortis as a result of the loss of ATP.

All too often glycolysis and the production of "lactic acid" is considered in isolation, without concern for the function of this enzymic process in living muscle, viz. to synthesize ATP from ADP and inorganic phosphate (Pi) in anaerobic conditions. In "fast" muscles, which are quantitatively the most important, the production of sufficient pyruvate for mitochondrial oxidation can be achieved by a small fraction of the glycolytic enzymes present; the large excess of these enzymes exists for anaerobic circumstances. Since the freely reacting adenine mononucleotides exist almost entirely as ATP, glycolysis can occur only as long as ADP is being produced by an ATPase system. Thus despite detailed investigations into the control of glycolysis by phosphorylase and phosphofructokinase activities by hormones[2] or by the relative amounts of other enzymes, ultimately the rate is controlled solely by the ATPases. This point is clearly demonstrated in Figure 1. A cell-free glycolysing system was made by putting together the necessary purified enzymes, and allowed to react with ATP, glycogen and creatine in the presence of the essential cofactors and buffers.



Creatine was phosphorylated, producing ADP which stimulated glycolysis to restore the ATP level. This process continued until most of the creatine had been phosphorylated; in Figure 1 the final stages of the creatine phosphorylation are still causing a slow acidification in the "No ATPase" example. With added ATPase a steady fall in pH occurred, and the rate of acidification was proportional to the amount of ATPase present. The ultimate pH's were around 5.6, comparable

to those found in meat. On the other hand, with no ATPase present the pH did not fall below 6.7, and the ATP level at 24 h was the same as at the start of the experiment.

It is commonly stated that glycogen is converted to lactic acid, and it is this acid which causes the fall in pH. As a simplified view of the overall process this is a satisfactory statement, but a detailed study requires a more sophisticated approach. Glycolysis, that is the conversion of glycogen to lactate with simultaneous phosphorylation of ADP, actually results in an alkaline change at high pH, and even at low pH does not produce one equivalent of acid for each lactate ion evolved. But as pointed out above, glycolysis cannot occur in isolation, it must be accompanied by a system for dephosphorylation of ATP, which itself results in the production of acid. The detailed equations for acid production are as follows:

Glycolysis

$$\text{Glycogen} + 3\ Pi^{(1+x)-} + 3\ Mg\ ADP^{-} \longrightarrow 2\ Lactate^{-} + 3\ Mg\ ATP^{2-} + (2-3x)\ H^{+}$$

ATPases

$$3\ Mg\ ATP^{2-} \longrightarrow 3\ Mg\ ADP^{-} + 3\ Pi^{(1+x)-} + 3x\ H^{+}$$

Sum

$$\text{Glycogen} \longrightarrow 2\ Lactate^{-} + 2\ H^{+}$$

(x = degree of ionisation of phosphate, depends on pH)

The overall result is that two equivalents of acid are produced for each glycogen glucose residue metabolised, but the relative amounts produced by the glycolytic process and by the ATPases vary widely with pH, as shown in Table 1.

pH	x	H^+ released by glycolysis	H^+ released by ATPase
7.4	0.8	-0.4	2.4
7.0	0.6	0.2	1.8
6.8	0.5	0.5	1.5
6.6	0.4	0.8	1.2
6.2	0.2	1.4	0.6
5.5	0.03	1.9	0.1

x = degree of ionization of inorganic phosphate

Table 1. Relative amounts of acid produced by glycolysis and by ATPases, per glycogen glucose residue metabolised to lactate.

In living muscle the phosphocreatine level is high[3], but falls steadily post-slaughter. At first this is the main mechanism by which ADP produced by ATPases is re-converted to ATP, but as the phosphocreatine level becomes smaller, glycolytic phosphorylation of ADP becomes more important.[4] Studies of the concentrations of glycolytic intermediates in beef muscle[4] and in rabbit, sheep and pig muscles as well as in the purified glycolytic system described above[5] show that at no time post-mortem do intermediates subsequent to the phosphofructokinase step accumulate significantly. This indicated that at no time is the ADP level rate limiting, and that the first reactions of the glycolytic sequence provide just enough of the substrates necessary to remove ADP at the rate it is formed. This is achieved through feedback control of both phosphorylase and phosphofructokinase activity by the ADP level. Phosphorylase in resting and in post mortem muscle is entirely in the b form, requiring AMP for activity. Phosphofructokinase is in the inhibited state due to the high ATP level, and requires AMP to relieve this inhibition. However, the amount of AMP required to activate these enzymes *in vitro* is substantially less than the reported levels of this nucleotide in resting or post mortem muscle (0.05-0.25 μmoles/g[4,6]) from which one must conclude that most is "bound" or "compartmentalized" - not

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freely available to the enzymes. A similar conclusion is reached if one considers the equilibrium constants of the creatine kinase and myokinase reactions. By a series of calculations, making assumptions about the concentration of free magnesium ions in muscle, and using reported equilibrium constants and binding constants for $Mg\,ATP^{2-}$ and $Mg\,ADP^{-}$, the "freely-reacting" concentration of ADP and AMP in muscle can be calculated, and are shown in Table 2 for a variety of pH conditions in post mortem muscle.

	Experimentally Determined (beef L. dorsi) μmoles/g			Calculated concentration of freely reaction nucleotide	
pH	PCr	Cr	ATP	mM ADP*	μM AMP
7.2	20	20	6.0	.08	.30
7.0	12	28	5.9	.09	.32
6.8	8	32	5.5	.09	.30
6.6	4	36	4.5	.09	.26
6.5	2	38	3.8	.09	.30
6.4	1	39	3.0	.14	.60
6.3	0.5	39.5	2.2	.18	1.0

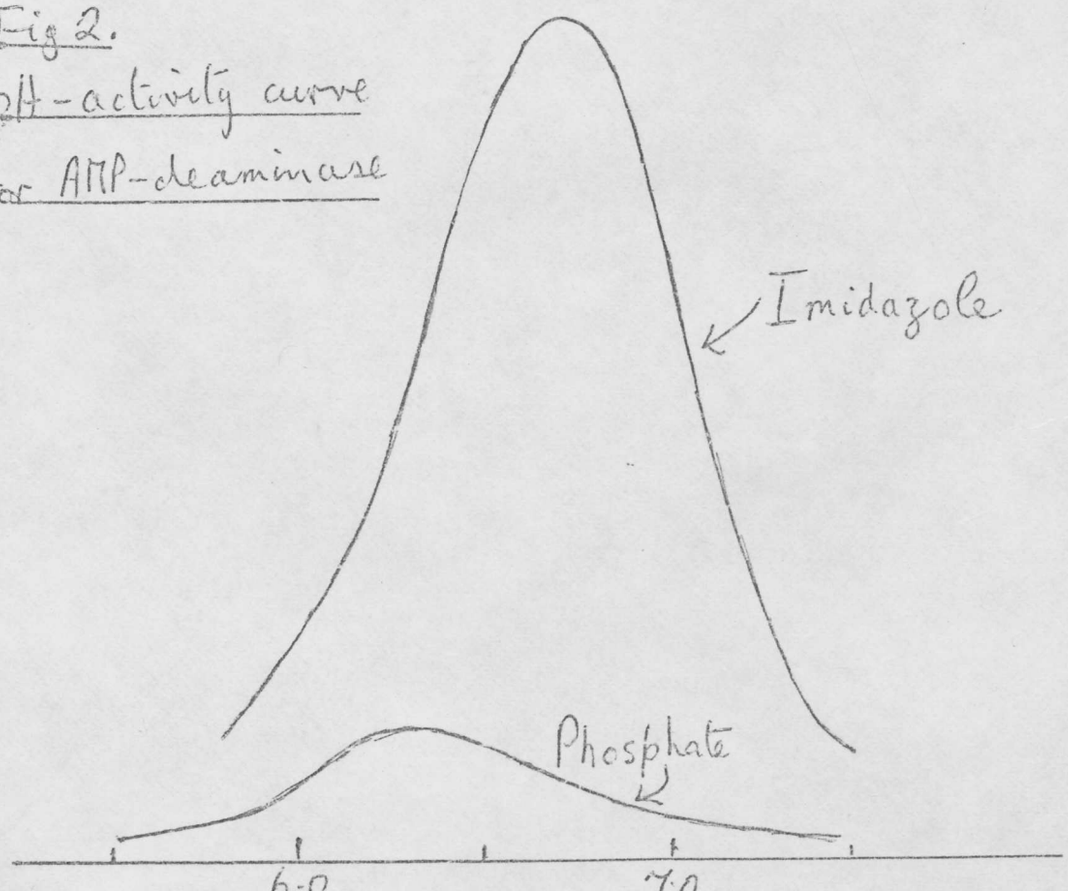
* ADP = sum of $Mg\,ADP^{-}$ and ADP^{3-}

Table 2 Calculated levels of freely reacting ADP and AMP in post mortem muscle

The AMP concentration remains fairly constant in the sub-micromolar range until below pH 6.5, when it rises abruptly. These calculated values are of the right order to account for the throughput of phosphorylase b in post mortem muscle.

The enzyme AMP deaminase is unable to act significantly on sub-micromolar concentrations of AMP. But as the pH falls, we see from Table 2 that the AMP level rises. Moreover, the specific activity of AMP deaminase increases as the pH approaches the optimum (in phosphate buffer) at 6.3 (Figure 2).

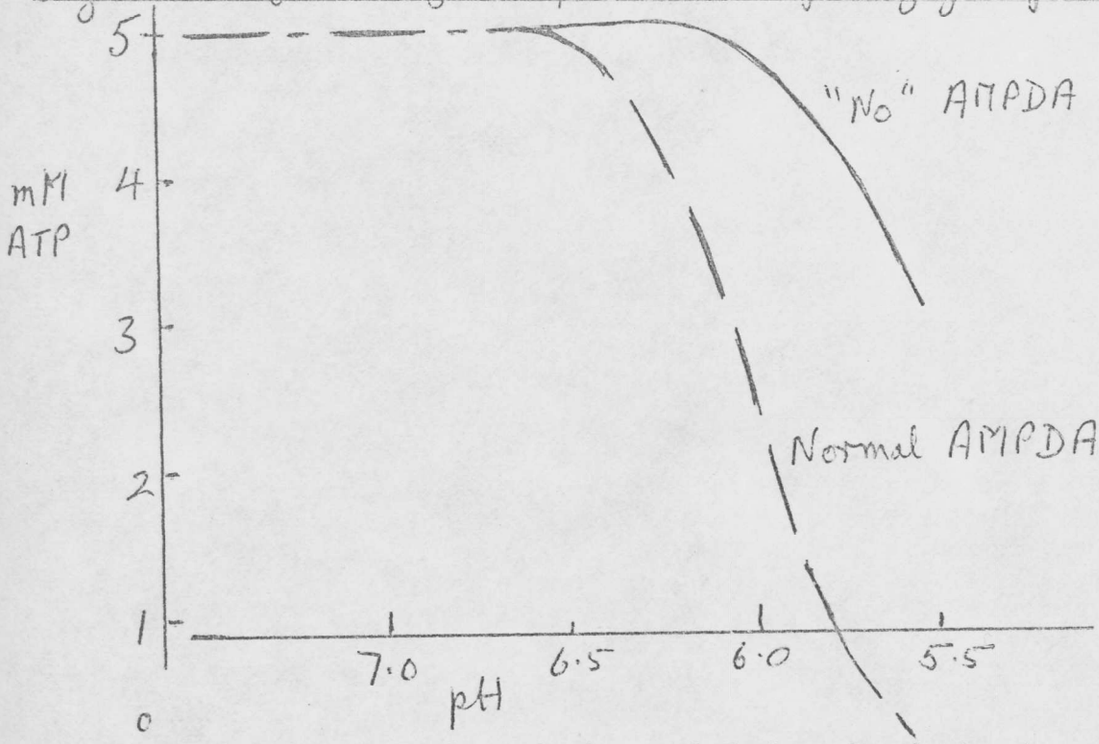
Fig 2.
pH-activity curve
for AMP-deaminase



This combination of increased specific activity and increased substrate level leads to a substantial rate of deamination around this pH; it is in the range pH 6.5-5.9 that IMP accumulates most rapidly in post mortem muscle. The action

of myokinase restores the lost AMP, at the expense of ADP, and the ADP is in turn restored by ATPases, lowering the ATP level. And so depletion of ATP with simultaneous build-up of IMP is due primarily to the activity of the enzyme AMP deaminase. Indeed if there were no AMP deaminase in muscle, the muscle might never lose its adenine nucleotides, and ATP would remain much longer, causing a delayed onset of rigor mortis and probably a lower ultimate pH. Using the isolated glycolytic system described above, an attempt was made to investigate this condition, by not including any AMP deaminase in the system. In practice it has proved difficult to obtain an ATPase free of AMP deaminase, but the predicted result does occur, as shown in Figure 3. In the system lacking deaminase, the ATP level remained at its initial level down to pH 6.0, and had only fallen to 50% of its initial value by pH 5.6.

Fig 3. Loss of ATP against pH in cell-free glycolysing system.



The ultimate pH of muscle is reached when glycolysis stops, and this occurs when there is no longer any freely reacting adenine mononucleotide in the muscle. In the cases of ultimate pH's above about 5.9, especially in examples of dark-cutting beef, glycolysis stops simply as a result of a complete utilization of glycogen. In normal meat with low ultimate pH's however, there is usually residual glycogen at the ultimate pH, and the exact determinant of the point at which glycolysis ceases is not so clear. Nevertheless it follows from the argument that the enzyme AMP deaminase is the sole cause of loss of adenine nucleotides that the variation in ultimate pH's found in normal meat could be due to differences in the activity of this enzyme during the post mortem pH fall.

Having described some theoretical aspects of post mortem glycolysis, I should like now to come to some experimental observations and try to explain these in terms of the theory described. pH-time curves for lamb muscle excised and placed at the indicated temperature are shown in Figure 4. The rates are in most respect similar to those found for beef sternomandibularis muscle[7] except that the ultimate pH of lamb tends to be 0.1-0.2 units higher. The rates decrease with decreasing temperature until about 10°; below this temperature the rate of pH fall down to pH 6.3 increases with decreasing temperature. Paralleling this phenomenon we observe cold-shortening of the muscle[8], the rate and extent of cold-shortening increasing as the temperature approaches freezing point. It is clear that the underlying cause of cold-shortening is also responsible for the accelerated pH fall, i.e. the accelerated ATPase activity at temperatures below 10°C

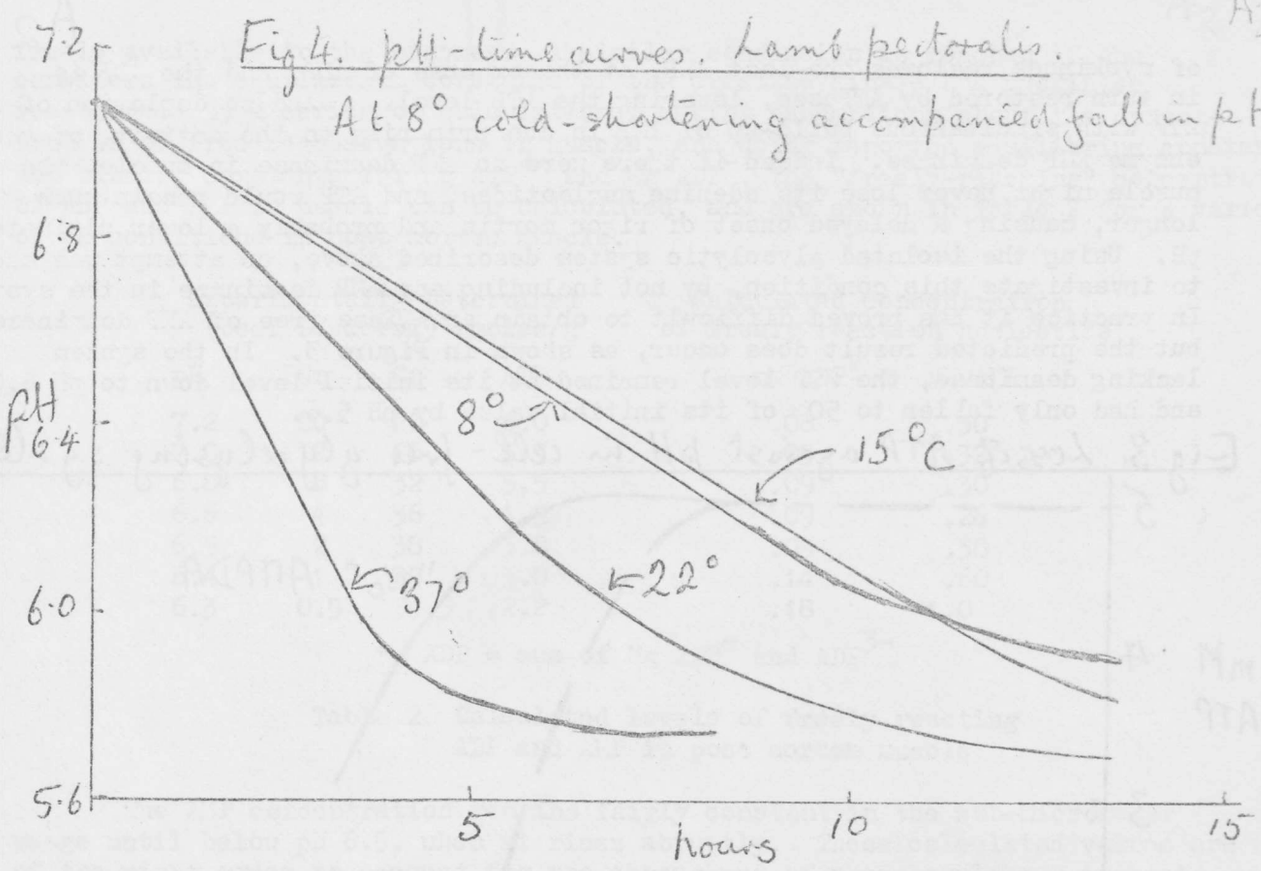


Fig 4. pH-time curves. Lamb pectoralis.
At 8° cold-shortening accompanied fall in pH

Below about pH 6.3, the rate of pH fall at low temperature decreases sharply to the sort of value expected by extrapolation from higher temperature, i.e. the excess ATPase activity associated with cold-shortening is no longer operating. Indeed cold-shortening does not occur if the muscle, having reached pH 6.3 at a higher temperature is then rapidly cooled. All evidence at present indicates that the underlying cause is a release of Ca^{++} ions from the sarcoplasmic reticulum. At these low temperatures the sarcoplasmic reticulum cannot operate sufficiently fast to prevent the cellular Ca^{++} concentration from rising above the critical value for myofibrillar contraction (causing shortening) and ATPase stimulation (causing the faster pH fall).

Another familiar abnormality in post mortem glycolysis is the PSE-pork syndrome. In Figure 5 are shown typical pH-time curves for PSE and normal pork.

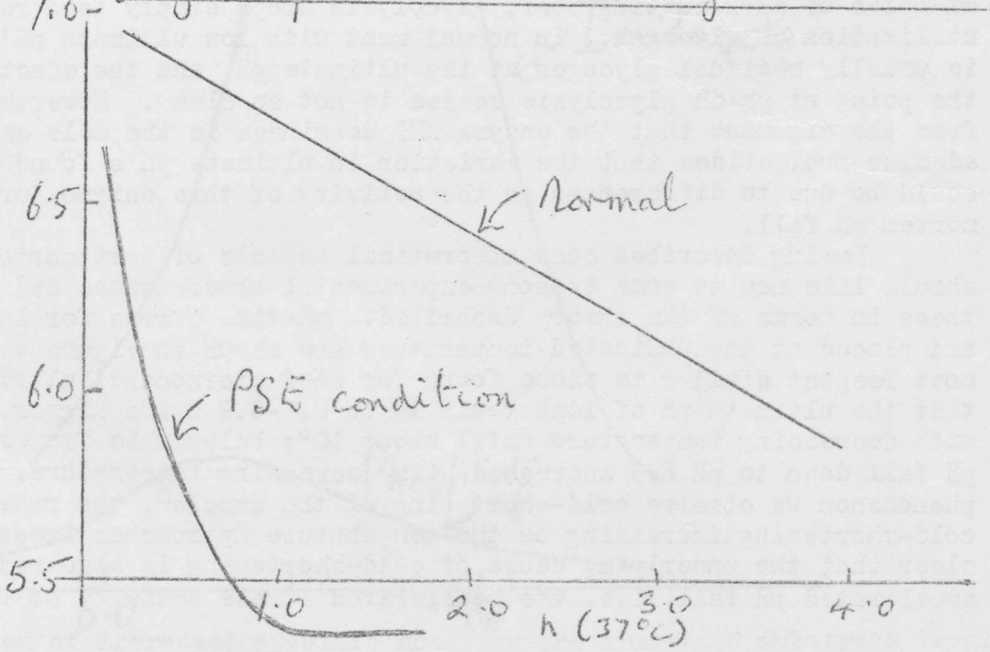


Fig 5. pH-time curve. Pig l. dorsi at 37°C

From the arguments above it is clear that the PSE muscle for some reason has a far greater ATPase activity in it than the normal muscle. Possible causes of this will be discussed in the next paper, but some significant features can be mentioned.

now. There is evidence that, as in the case of cold-shortening, the increased ATPase is myofibrillar in origin caused by a super-critical Ca^{++} concentration. Muscle contains an enzymic system which responds to Ca^{++} by increasing the preparedness of glycolysis to cope with impending demands for ATP, namely the enzyme complex for converting phosphorylase b to a. Apart from all the detailed subtleties of this system including hormonal response, it is apparent from recent work that phosphorylase a is only produced in the presence of Ca^{++} 9, 10. Phosphorylase a reveals itself in a higher rate of phosphorylase activity than of phosphofructokinase, causing accumulation of the intermediate hexose monophosphates. From Table 3 it is clear that PSE pig muscles do have phosphorylase a present, which in turn implies a cellular Ca^{++} level high enough to cause the phosphorylase b to a conversion.

	Mean time to pH 6.0 at 37°	Hexose Monophosphate at pH 6.3, μmoles/g	Fructose Diphosphate at pH 6.3, μmoles/g
Very Fast (PSE)	27 min	17 ± 1.2	0.35 ± 0.1
Fast (PSE)	56 min	13 ± 2.0	0.25 ± 0.1
Slow	230 min	4 ± 1.5	0.05 ± 0.03

Table 3 Hexose phosphate levels in pig muscle post mortem

Although myofibrillar ATPase is probably involved in the increased rates observed in cold-shortening and PSE muscle, there are many other ATPase systems present which might be responsible for normal basal metabolic rates. Such systems are listed below.

Particulate ATPases
- Myofibrils
- Sarcoplasmic Reticulum
- Mitochondria
- Membranes

Soluble ATPases
- Cyclic: kinase/phosphate systems
  glycogen synthetase/phosphorylase
- "Water kinase" side reactions of kinase enzymes

Table 4 ATPases in muscle

In living muscle there must be a slow breakdown of ATP in maintaining ionic balances both across the cell membranes (e.g."sodium pump") and within the cells (e.g. "calcium pump" of sarcoplasmic reticulum). Isolated sarcoplasmic reticulum exhibits *in vitro* a maximum ATPase rate far in excess of that needed to account for all of the normal post mortem ATPase; although the conditions for the maximum rate was never found post morte, this fraction could account for much of the observed resting ATP turnover. Mitochondrial ATPase may also contribute to the "particulate" ATPases. Other systems can be classed as soluble ATPases, in that they are associated with soluble proteins rather than with structural components. Obviously a true soluble ATPase, catalysing a simple dephosphorylation of ATP without coupling to any energy requiring process would not be of any use to a cell except to keep it warm, and it is unlikely that such an enzyme would exist in mammalian muscles. Nevertheless there are cyclic systems of the form:

$$X + ATP \xrightarrow{\text{kinase}} X - P + ADP$$

$$X - P \xrightarrow{\text{phosphatase}} X + Pi$$

$$\text{net result: } ATP \longrightarrow ADP + Pi$$

Enzymes to catalyse such systems are known where X = glucose, fructose 6-phosphate, phosphorylase b, and 3-phosphoglycerate (in the latter case the "phosphatase" consists of two enzymes). More elaborate is the system involved in glycogen synthesis, which coupled with phosphorylase leads to a net ATPase, with five enzymic reactions involved;

UDPG + $(Glycogen)_n$	⟶	UDP + $(Glycogen)_{n+1}$
UDP + ATP	⟶	UTP + ADP
UTP + Glucose 1-P	⟶	UDPG + PPi
PPi	⟶	2 Pi
$(Glycogen)_{n+1}$ + Pi	⟶	$(Glycogen)_n$ + Glucose 1-P
Net result: ATP	⟶	ADP + Pi

Even more elaborate sequences of reactions exist, but the ones above are more important as the quantities of enzymes catalysing the reactions are quite large. However, each of these systems has controlling factors so that they do not cycle round wastefully. For instance in the conditions where the glycogen synthetic complex is active, phosphorylase is largely inactive, and whereas phosphofructokinase is activated by traces of AMP, fructose diphosphatase is equally affected by being inhibited by sub-micromolar AMP concentrations. Indeed, adding muscle fructose diphosphatase at five times the normal concentration to the purified glycolytic system, in the absence of any ATPase, did not result in any more ATP breakdown, indicating that this particular cyclic system does not operate as an ATPase post mortem.

The final possible source of ATP breakdown is by side reactions of other enzymes, in general, kinases. No enzyme is perfect, and several kinases have been shown to exhibit trace amounts of ATPase activity ("water kinase")[11,12]. From known figures it is possible that at least 20% of post mortem ATPase activity in normal muscle is due to the ATPase activities of the enzymes creatine kinase, phosphofructokinase, pyruvate kinase, phosphoglycerate kinase and myokinase. Although these are only between 10^{-5} and 10^{-4} times the true kinase activities, the quantities of the enzymes present makes the total significant.

In conclusion, it can be considered that the rate of post mortem glycolysis is controlled by the ATPases include sarcoplasmic reticulum and soluble systems, and contributions may also be made from mitochondrial and myofibrillar ATPases. In abnormal conditions, when the rate is much higher (PSE muscle) or higher than expected (cold-shortening muscle), these are supplemented by Ca^{++}-stimulated myofibrillar ATPase, which has a potential of up to 500 times the normal post mortem rate of ATP breakdown observed.

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