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The biochemistry of rigor mortis and cold-contracture

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The aim of this report is to describe the general biochemical background of the process of rigor mortis, with particular reference to the phenomenon of cold contracture. For this purpose it is necessary to recapitulate briefly our fundamental knowledge of the contractile process and of the ATP-resynthetic mechanisms in muscle. For more details of these processes readers may like to refer to my chapter in the new edition of the Structure and Function of Muscle which will be published this year.

THE FUNDAMENTAL BASIS OF THE RIGOR PROGRESS

I. The physical basis

The physical basis of rigor is the large change in the stretch characteristics of muscle, often accompanied by active shortening of the fibres. Pre-rigor muscle can be easily stretched to upwards of 120% of its slack length by loads of 50 gcm^{-2}, and will completely recover from the stretch fairly quickly after removal of the load (Bate-Smith, 1939). Muscle in full rigor, on the other hand, can be stretched by less than 1% by similar loads (i.e. to 101% of the slack length). Increasing the load tenfold (to 500 gcm^{-2}) only marginally increases the stretch, perhaps to 1.5 to 2%; this additional stretch is also reversible on removal of the load. Attempts to stretch the rigor muscle further result in fracture of fibres and impairment of recovery.

These remarks apply to muscle stored at 10° or lower for not longer than 36 hrs post-rigor. Longer storage results in increased and irreversible stretch even to loads of 50 gcm^{-2}. This loss of strength is greatly accelerated at higher temperatures of storage (25 - 37°), the response to small loads increasing almost back to the pre-rigor level of 30% of the slack length, but becoming completely irreversible (Davey and Gilbert, 1969; Bendall, 1973).

The large decrease in stretchability during rigor strongly suggests that longitudinal elements within the muscle fibres, previously able to slide freely over each other, have "seized up" and become cross-linked to one another. Indeed, the sliding filament theory of muscle contraction (Hanson and Huxley, 1955; cf. Bendall, 1969) provides an elegant explanation of how this occurs.

The muscle fibre is a very long multi-nucleated cell about 50 μm in diameter, bounded on the outside by the sarcolemma and filled with 1000 or so fibrils, themselves delineated by the longitudinal elements of the sarcoplasmic reticulum (SR) which enwraps them and which connects with the external sarcolemma by means of its triad system and its transverse elements (cf. Bendall, 1969). Within the fibrils are the primary filaments of actin (thin) and myosin (thick) which interdigitate with one another (see fig. 1). The actin filaments are in fact double helices of globular actin monomers, and the myosin filaments aggregates of long myosin molecules, bound together by their tails with their double-heads protruding. These heads contain the ATP-ase sites and are also the means by which the myosin filament can cross-link to actin monomers in the thin filament. Each myosin filament is surrounded by six actin filaments and each actin by three myosin filaments (fig. 1C).

In the resting state, the fibrils are replete with ATP in the form of its Mg chelate ($MgATP^{2-}$) which acts as a plasticiser to keep the two sorts of filament apart and to prevent cross-linkage. $MgATP^{2-}$ is however in continual dynamic equilibrium with $MgADP^{1-}$, because it temporarily attaches itself to the enzymic sites of myosin (myosin heads) and is then rapidly split to $MgADP^{1-}$ and P_i^{2-}. These products under relaxing conditions dissociate only very slowly, thus restricting the resting turnover of ATP to very low rates. For this reason, the myosin sites (see fig. 1a) are mostly saturated with $MgADP^{1-}$ in the resting state (White, 1970; Lymn and Taylor, 1971; Trentham et al., 1972).

Two other proteins play a vital role in rest and contraction, tropomyosin and the troponin complex. Long thin molecules of tropomyosin lie in the grooves of the actin helices and attached to them at intervals are troponin complexes consisting of three globular proteins of low molecular weight (Schaub and Perry, 1971; Graeser and Gergely, 1971). In the resting state, when there is plenty of $MgATP^{2-}$ present, the troponin complexes have the peculiar property of being able to repel the myosin heads (saturated with $MgADP^{1-}$) and prevent them from attaching to actin monomers. Thus actin and myosin filaments can slide freely over each other during rest; they do not, however, seem to have any ability to relax back spontaneously to their original

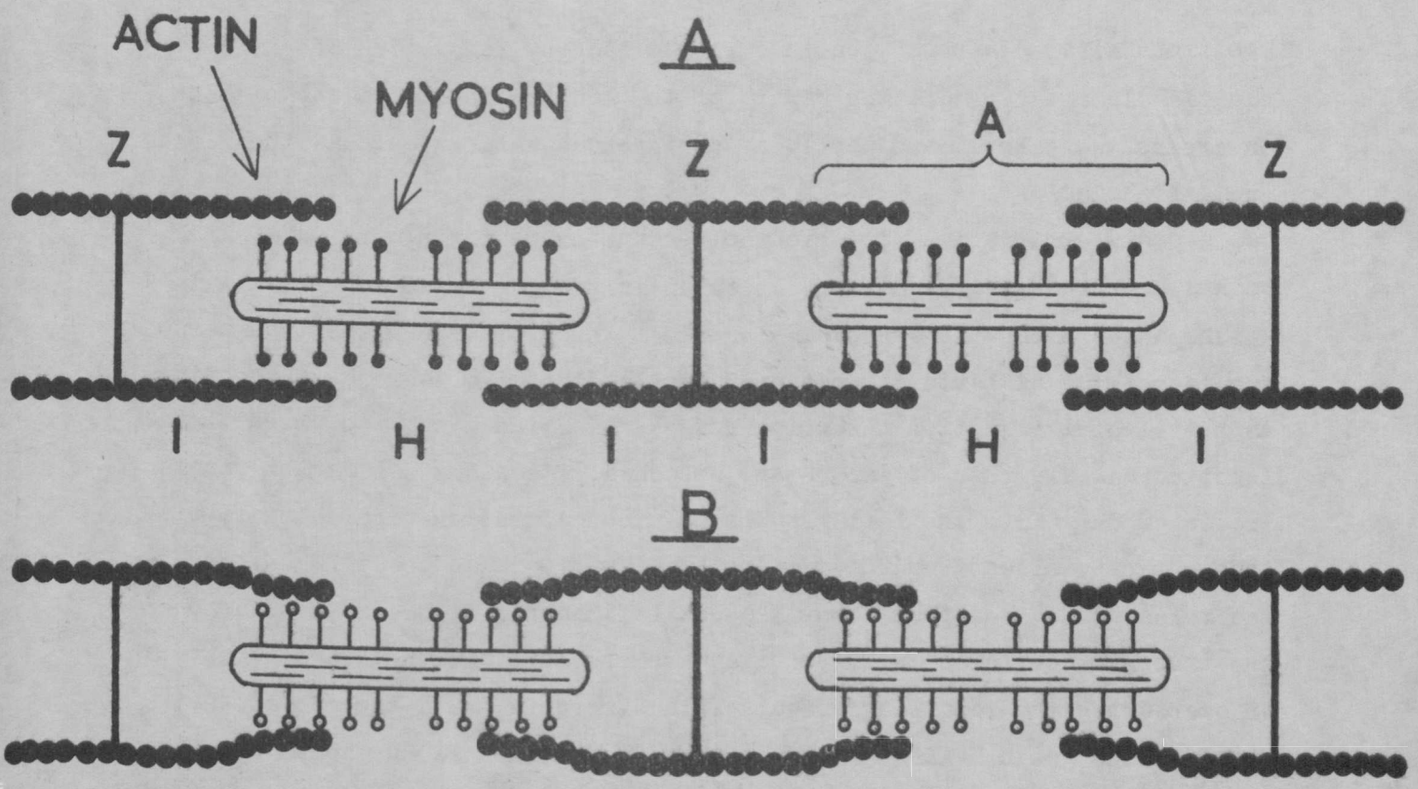

FIG. 1

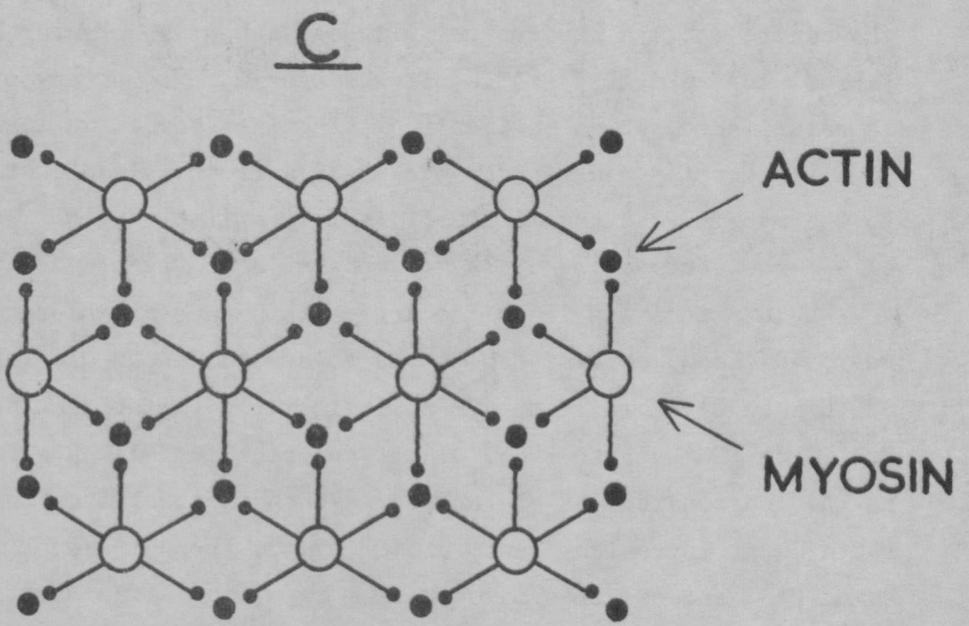

Fig. 1 Sliding filament model of sarcomeres, showing actin and myosin filaments:

A. pre-rigor, resting sarcomeres; myosin heads (dark) with $MgADP^{2-}$ attached;

B. rigor sarcomeres; myosin heads empty of $MgADP^{2-}$;

C. cross-section in region of overlap (resting).

positions after a stretch, but are pushed back by relaxation of parallel elastic elements, consisting partly of the longitudinal elements of the SR around the fibrils and partly of collagenous networks around the fibres and fibre bundles.

Normal contraction, to which cold-contracture is in many ways akin, occurs as a result of a nervous stimulus initiating an action potential which passes along the sarcolemma and into the fibre along the transverse tubules of the SR (situated often at the level of the Z-discs of the fibrils). As the electrical action potential passes the triad system to which the longitudinal elements of the SR are attached, it causes release of minute traces of Ca^{2+} from the latter. The Ca^{2+} concentration within the fibril then abruptly rises from its resting level of 10^{-8}M to about 10^{-6}M. As the released Ca^{2+} diffuses over the actin filaments it becomes bound to the troponin complexes in these filaments and this has the dramatic effect of overcoming the repelling effect of the troponin, with the result that myosin heads (with $MgADP^{1-}$ bound to them) can attach to any actin monomers within range. This causes a 300-fold increase in the rate of release of $MgADP^{1-}$ from the myosin sites, and brings about a kind of flicking motion of the myosin heads which thereby shift the actin filaments a step towards the centres of the sarcomeres, with consequent shortening of the whole fibre. As the $MgADP^{1-}$ falls off the myosin, a temporary myosin-actin link is established, which is identical with that formed during rigor. But this link is abruptly and rapidly broken as a fresh molecule of $MgATP^{2-}$ attaches to the myosin head and is itself rapidly split to $MgADP^{1-}$ and P_i; this allows the contractile cycle to repeat itself (Lymn and Taylor, 1971).

Relaxation sets in as the ionic status quo at the sarcolemma is restored, because this enables the so-called Ca-pump in the triads of the SR (driven by energy from ATP-splitting) to start operating and pump back Ca^{2+} from the neighbourhood of the contractile filaments into its storehouse in the longitudinal SR elements. As soon as the intra-fibrillar Ca^{2+} is reduced to its resting level of 10^{-8}M, the troponin complexes lose their bound Ca^{2+} and can once more exert a repelling effect on the myosin heads, so that the muscle relaxes (providing plenty of ATP is still available).

In a very prolonged tetanus, the muscle will eventually run out of a supply of ATP, so that the system "seizes up", loses its stretchability and passes into rigor, because there is then no means of breaking up the myosin-actin linkage, formed in the last step of the contractile cycle. We can envisage this rigor state as cross-linkage of myosin heads to actin monomers wherever the two are in range of one another (fig. 1B).

The normal rigor process is physically identical with the above, but takes place on a quite different time scale, because the musculature after slaughter of an animal is usually in the fully relaxed state with a good supply of ATP. ATP is then turning over only very slowly, as we said earlier, at a rate of about 1/300th of the contractile rate. Even this low rate will, however, eventually exhaust the ATP supply and the systems which resynthesise it, and rigor will then inevitably occur. The rigor process is irreversible in the strict sense, unless MgATP can be made available, as in artificial glycerolated muscle models, where rigor can then be reversed in the additional presence of Ca-chelators such as EGTA (Bendall, 1969).

II. Biochemical changes during the rigor process

a) ATP-resynthetic mechanisms

The most significant feature of rigor in muscles from well-fed and rested animals is that the ATP level (5 - 8.5 μ.mole g^{-1} in mammals, depending on the species) remains high and constant for a considerable time, yet it is being continually split at the ATP-ase sites of myosin, albeit slowly. It follows that the ATP-resynthetic systems of the muscle are able initially to rephosphorylate ADP as quickly as it dissociates from the myosin sites. In the post-mortem muscle, where the last traces of oxygen have been used up within 3 min. of slaughter and bleeding, these systems consist of the creatine kinase reaction and the reactions of anaerobic glycolysis. The first of these can be formulated as:

1) $MgADP^{1-} + PC^{2-} + H^{1+} \longrightarrow MgATP^{2-} + \text{creatine}$

Note that this reaction absorbs 1 hydrogen ion and hence results in an alkalisation. However, the original splitting reaction neutralises this, since it has already produced 1 H^{1+}:

2) $MgATP^{2-} + H_2O \longrightarrow MgADP^{1-} + P_i^{2-} + H^{1+}$

Accompanying the creatine kinase reaction are the complex reactions of glycolysis which accept ADP and rephosphorylate it in competition with the former. In these reactions 1 glucose unit of glycogen is broken down to 2 mole of lactate^{1+} with simultaneous resynthesis of 3 mole ATP from 3 mole ADP and 3 mole P_i. During this process 1 H^+ is actually absorbed per 2 mole lactate^{1-} formed, but for this to happen 3 mole ATP must have already been broken down to 3 mole ADP in reaction 2, thus producing 3 H^{1+}. Hence the overall net effect is for 2 H^{1+} to appear per glucose unit used up (i.e. per 2 lactate^{1-} produced), (Scopes, 1971).

The balance between reaction 1 and glycolytic resynthesis, while the PC level is still high, is about evenly divided, but glycolysis rapidly gains ascendancy as soon as PC has fallen from its initial level of 12 - 23 μ.mole g^{-1} to about 3 μ.mole g^{-1}. Glycolysis is relatively inefficient, with the result that when it is unsupported by the creatine kinase reaction, the ATP level begins to fall; for this reason, PC can be regarded as an ATP-buffer (Scopes, 1971). At this stage ATP is irretrievably lost, because of the combined operation of the myosin ATP-ase, myokinase and AMP-amino-hydrolase reactions which can be formulated as:

3) $MgATP^{2-} \longrightarrow IMP^{2-} + 2P_i^{2-} + Mg^{2+} + NH_4^{1+} + H^{1+}$

The ultimate fate of IMP is to be degraded to hypoxanthine and ribose, but this is a comparatively slow process and takes place mostly in the post-rigor period (Lee and Newbold, 1963).

Summarising, we can say that the main biochemical changes during rigor are the following:

a) the splitting of MgATP at the myosin enzyme sites;

b) the slow release of one product, ADP, which diffusing away from the sites encounters creatine kinase plus PC, leading to its rapid phosphorylation to ATP via equation 1;

c) the diffusion of the other product of splitting, P_i, to the sarcoplasm surrounding the fibrils where encountering phosphorylase, it phosphorylates a glucose unit of glycogen to produce glucose-1-P and thus to start the glycolytic sequence. The latter can reach completion (formation of lactate) only if some of the ADP released from the myosin sites can reach re-phosphorylating enzymes lower down in the sequence.

d) the gradual exhaustion of the PC supply and the taking over of resynthesis entirely by glycolysis, which being rather inefficient allows escape of some ADP to myokinase. The result is the deamination and dephosphorylation of the latter to IMP, P_i and NH_4^{1+} through equation 3.

e) the final exhaustion of ATP and the setting of the muscle in rigor, via the formation of cross-links between actin monomers and the now ADP-depleted myosin heads (fig. 1B).

There are two other consequences of ATP depletion: the first is that as the ATP level falls anywhere in the neighbourhood of an active site to less than about 0.1 μ.mole g^{-1}, that particular myosin head spontaneously goes through the flicking motion characteristic of contraction (White, 1970). Consequently, the localised rigor process is always accompanied by an attempt

by the muscle to shorten. The degree of shortening will entirely depend on how many sites at any instant become depleted of ATP. We shall discuss this aspect in more detail later.

Another totally unexpected effect of ATP depletion has recently come to light. Because the Ca-pump of the SR is dependent on energy from ATP splitting, depletion of ATP results in the slow release of Ca^{2+} ions from their storehouse in the longitudinal elements of the SR. Thus there is an irreversible increase in the intrafibrillar Ca^{2+} concentration, sufficient to activate a moderately specific proteinase, recently discovered by Busch et al. (1972), which has the property of attacking the Z-disc structure of the fibril. Disappearance of the Z-discs had already been noted during the conditioning of meat (Davey and Gilbert, 1969) and was taken to account for the gradual resolution of the rigor muscle's high resistance to stretch (mentioned in Section I above). We thus now have an almost complete explanation of this phenomenon.

b) <u>the biochemical and physical pattern of rigor at constant temperature.</u>

The biochemical pattern of rigor in resting muscles, well stocked with glycogen, is remarkably uniform over a wide range of conditions. The general pattern can be well illustrated by the example of a rabbit psoas muscle passing into rigor at 38° (fig. 2). Here we have chosen to show lactate production in terms of the pH change to which it is inversely and linearly related (see table 1).

We note from fig. 2 how the level of ATP stays nearly constant at about 8 μ.mole g^{-1} until the PC level has fallen below about 4 μ.mole g^{-1} and then falls on an S-shaped curve as it is gradually dephosphorylated and deaminated to IMP (equation 3). While the PC level is still falling, the pH also falls at a steady rate until the former is somewhat depleted, when there is a noticeable increase in the rate (at about pH 6.7 in this case). This change of rate is a constant feature of rigor in resting muscles and is particularly well marked in beef LD and sternomandibularis (STM) muscles. It is due to the fact that the ATP-turnover rate at the myosin sites is nearly constant throughout the whole course of rigor (39 μ.mole ATP $hr^{-1}g^{-1}$ in rabbit psoas and about 32 μ.mole $hr^{-1}g^{-1}$ in beef LD and STM at 38°). Consequently when resynthesis of ATP from PC fails, glycolysis increases in rate to cope with the increasing availability of ADP to it.

The physical change, i.e. the loss of stretchability during rigor, is shown in fig. 2 as increasing resistance to stretch (R_S) (= 1/relative stretchability). At this temperature, it sets in at a rapid rate as soon as ⅓ to ½ of the ATP has been lost and is completed when only traces of ATP

FIG. 2

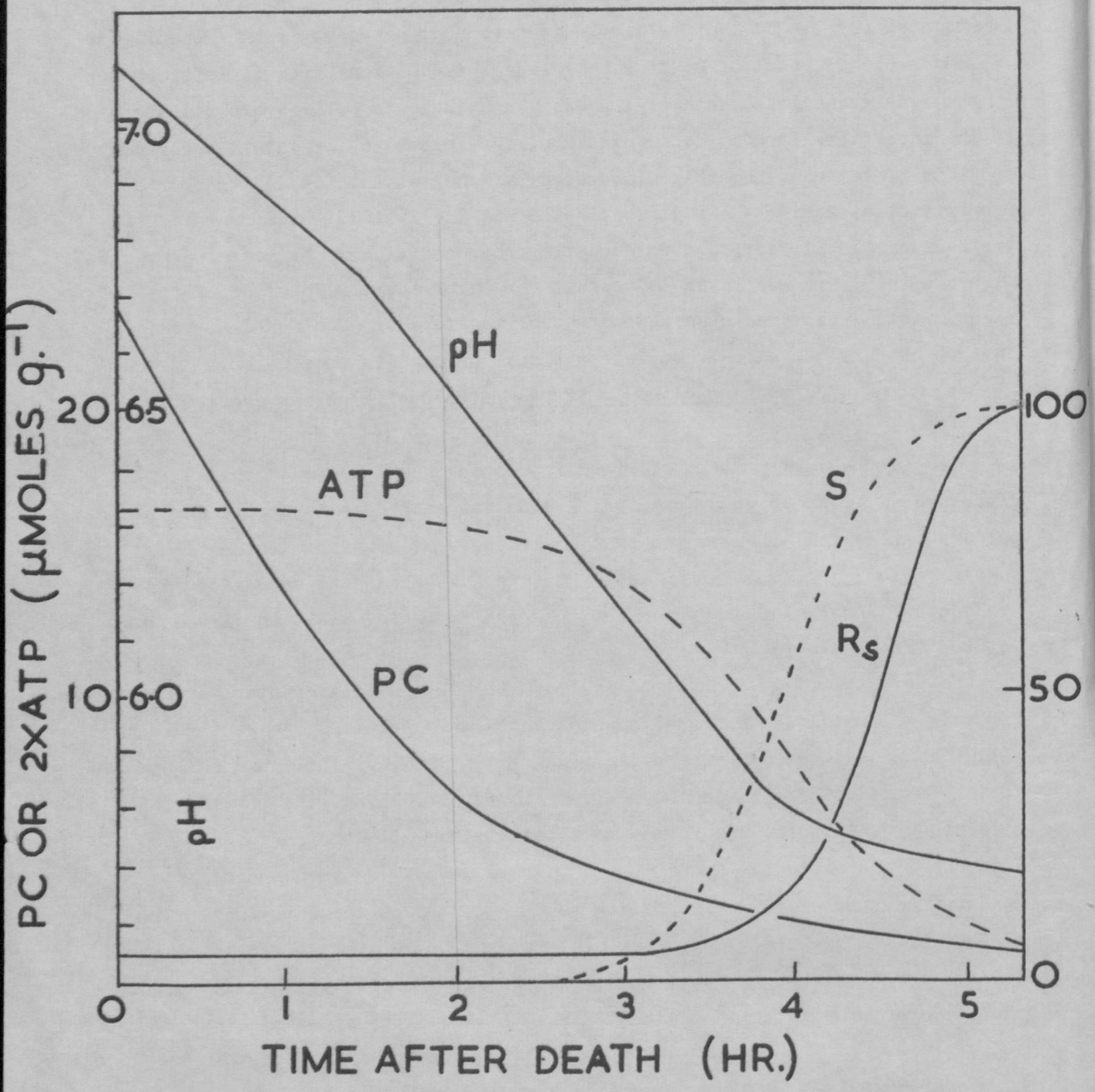

Fig. 2. Pattern of rigor changes in rabbit PS muscle at 38°.
S = shortening: R_S = resistance to stretch.

remain in the muscle. About ½ hr before the increase in R_S has begun, the muscle begins to shorten under the intermittent load imposed on it during the measurement of R_S (see Bate-Smith and Bendall, 1947 and Briskey et al. 1962, for methods of measuring R_S). At this temperature, under an intermittent load of 50 $g.cm^{-2}$, shortening (S) can amount to upwards of 25% of the initial muscle length.

There can be little doubt that the rigor change is a statistical process in the sense that fibres and parts of fibres become depleted of ATP at varying times. This is deducible from the fact that neither rigor shortening nor loss of stretchability can be induced in relaxed model fibre systems until the ATP level is reduced to less than 0.1 μ.mole g^{-1} (White, 1970). Thus in the example in fig. 2, it cannot be true that, when half the initial ATP level has been destroyed (i.e. when 4.1 μ.mole remains per g), all the sarcomeres in the muscle are half depleted of ATP; if this were the case, no shortening or loss of stretchability would occur anywhere in the muscle. Rather it must be so that at this stage ATP is almost entirely lacking in half the sarcomeres, just as that ¾ of the sarcomeres lack ATP when ¾ of the total ATP has been lost, and so on. This also explains the observed fact that active rigor shortening runs almost anti-parallel to the declining ATP level, providing the load on the muscle is not large (cf. Bendall, 1951).

The rigor pattern is drastically modified if much ponnistelu struggling occurs during slaughter, because this leads to loss of PC and a more or less marked fall in pH. For example, in rabbits killed by a blow on the neck there is a severe death struggle, which often reduces the initial pH to 6.5 or lower and the initial PC to less than 6 μ.mole g^{-1}, with some loss of ATP. The effect is to curtail the rigor diagram in fig. 2 as if it started at about 2 hr post-mortem instead of at 0 hr. Loss of stretchability then sets in slowly from the moment of death, because quite a number of sarcomeres are already depleted of ATP, due to the struggling; rigor is completed within 3 hr post-mortem. This type of pattern is frequently met with in pig LD muscles and is quite common in beef psoas and tensor fasciae latae (TFL).

In effect the struggle we described above expends about 0.6 pH units worth of glycolytic potential (= 39 μ.mole lactate g^{-1} = 19.5 μ.mole glucose units of glycogen = 3.2 mg glycogen g^{-1}). A similar expense of glycolytic potential can be brought about by exhausting the living animal or exposing it to stress conditions or by injecting it intramuscularly with adrenalin. Providing the exhausted animal is killed with the minimal struggle, the muscles will nevertheless have high initial levels of pH, PC and ATP, but the glycogen store will be less, and the ultimate pH will be raised from about 5.6 to 6.2 for this reason. This has a much less drastic

effect on the rigor pattern than a violent struggle at slaughter does, cutting off only about 1 hr from the time for completion of rigor at 38°. Cases of this type have been discussed by Bate-Smith and Bendall (1949) and Bendall (1960 and 1973, in press). In extreme cases stress may lead to much greater depletion of glycogen and to ultimate pH values higher than 6.8. In red beef and pig muscles such high ultimate pH's cause the meat to be more translucent than normal so that light penetrates much deeper into the surface and gives the meat the well-known dark-cutting appearance. In resting, well-fed animals there are, at any given temperature, fairly strict relations between the pH, on one hand, and the PC and ATP levels on the other. In the rabbit psoas at 38°, for example, $\frac{1}{8}$ of the ATP is lost at pH 6.3 and $\frac{1}{2}$ at pH 5.9. At 38°, the pig LD is similar, even in the cases of extremely rapid ATP-turnover and pH fall which give rise to PSE meat. At 2° in contrast, beef LD, TFL, STM AND D muscles (see table 1 for abbreviations) lose about the same amounts of ATP (1 and 4 μ.mole g^{-1}, respectively) at pH 6.5 and 6.2, probably because the resynthetic mechanisms are not so efficient at the lower temperature.

Table I summarises the initial PC and ATP levels, the ultimate pH and the buffering capacity (B) in the muscles of 4 species of mammal. There are large species variations in initial PC and ATP levels, the rabbit PS showing the highest levels of both and the beef D the lowest. Frog sartorius, by the way of contrast, has a high initial level of PC (24 μ.mole g^{-1}), but very low ATP (3.6 μ.mole g^{-1}).

Table I. Initial PC and ATP levels, ultimate pH values and buffering capacity (B) in various muscles of mammals. All values corrected to an initial pH of 7.1, where necessary.

Muscle[a]	Initial contents in μ.mole g^{-1} of: PC	ATP	Ult. pH	B. (lactate formed) μ.mole pH^{-1} g^{-1}
Beef LD	19	6.1	5.58	58
TFL	21	5.8	5.66	-
STM	17	5.2	5.77	54
D	11	4.8	5.80	-
Pig LD (LW)	19	6.6	5.60	72
Lamb PP (6 m)	13	5.9	5.70	58
Rabbit PS	23	8.1	5.65	65

a. LD = longissimus dorsi; TFL = tensor fasciae latae; STM = sternomandibularis; D = diaphragm; PP = pectoralis profundus; PS = psoas major.

THE EFFECT OF TEMPERATURE ON THE RIGOR CHANGES: COLD- AND THAW-CONTRACTURE

1. The general effect of temperature

The main effect of temperature on the biochemical pattern of rigor is that to be expected in general for any chemical reaction: the overall rate falls as the temperature is lowered from 38 to 5°. However, the rigor process is highly anomalous because it has a nearly constant temperature coefficient ($Q_{10°}$) of 1.7 from 38 to 25° only, but below this temperature $Q_{10°}$ decreases rapidly, reaching zero at about 5° in the red muscles of beef, pig, sheep and rabbit and at about 0° in the white muscles of the rabbit. In the first type of muscle, $Q_{10°}$ becomes increasingly negative below 5°, with the result that at 2° the rates of change have become as high as at 15°.

As we have pointed out, the fundamental rate-determining process is the rate of ATP turnover by whatever ATP-ase may be active (normally the myosin enzyme). This parameter can be calculated if the rates of pH fall (V_{pH}) and of PC and ATP loss (V_{PC}, V_{ATP}) are known. The rate of ATP turnover (V_A) is then given by:

4) $$V_A = V_{PC} + \underline{2}\, V_{ATP} + \underline{1.5}\, BV_{pH}$$

where B = μ.mole lactate produced per pH g^{-1}.

Table II shows the relative rates of ATP turnover (V_A) at temperatures from 38 to 2°, taking the rate at 38° as 100. The lowest line in the table shows the ideal decline in the rate if $Q_{10°}$ were constant at 1.7.

Table II Relative rates of ATP turnover in rabbit and beef muscles at temperatures between 38 and 2°.

Temp.	38	35	30	25	20	15	10	2
Rabbit (PS)	100	83.3	61.4	49.3	41.7	36.4	32.4	28.0
Beef (STM)	100	83.3	61.4	49.3	44.2	39.4	35.8	38.5
Ideal	100	84.0	62.9	46.3	33.8	24.4	17.4	9.8

It can be seen at a glance how anomalous the actual rates become below 25°, so that at 2° the observed rate in the rabbit psoas is 3x the ideal and that for beef STM 3.9x.

Now these anomalies cannot be explained if ATP turnover throughout the temperature range is solely determined by the slow, resting MgATP-ase on the myosin sites, because this enzyme shows no anomalies and has a constant Q_{10° of 1.7 from 38 to 0° (Bendall, unpublished observations). Hence as soon as anomalies appear some additional ATP-ase must be activated. The clue to the nature of this ATP-ase is given by the phenomenon of cold-contracture (Locker and Hagyard, 1963) which sets in in most muscles of the red type as the temperature is reduced below 11° and appears to reach a maximal rate at about 2.5° (Bendall, 1973) or perhaps lower (Lacourt, 1972).

Contractures can be produced, as far as we know, only if the actin-myosin contractile ATP-ase is activated, which happens when the Ca^{2+} concentration in the intra-fibrillar space rises from 10^{-8} towards 10^{-6}M, due to release of Ca^{2+} from the triads of the SR, as we showed in the first section. Hence it has been deduced that this is what occurs to an increasing extent as the temperature is reduced below 25°, the Ca release only reaching a critical contractile level below 11° (Newbold, 1966; Horgen et al., 1972). This indeed seems a highly plausible explanation of the anomalies, particularly if the Ca-pump of the SR has the extremely high Q_{10° which some research has indicated (Hill, 1972). If this were so, the pump would work extremely slowly at low temperature and so would be unable to cope with the inevitable passive diffusion of Ca^{2+} out of the triads.

That Ca-release is implicated in cold-contracture can also be concluded from results obtained during thaw- contracture which is more rapid and energetic than cold-contracture but has features in common. In thaw-contracture, the muscle, previously frozen in the pre-rigor state, is thawed out more or less rapidly, and as the temperature rises through the critical range of 0 to 10°, it contracts vigourously. As the contracture starts, there is a marked and rapid change in the rate of Ca-flux across the sarcolemmal membrane (Kushmerick and Davies, 1968) which later reverses; the reversal coincides with the spontaneous relaxation phase seen in loaded muscles, thawing rapidly (Bendall, 1960). Thus the Ca-pump is able to recapture Ca^{2+} as soon as the muscle temperature reaches about 15°. The same must apply to cold-contracture as well, which is reversible by merely raising the temperature above 11°, providing the muscle pH is above 6.5. In rabbit semitendinosus, for example, this cycle of cold-contracture and relaxation can be repeated at least 3 times by raising or lowering the temperature between 2 and 15°.

II Cold-contracture and rigor shortening compared.

In contrast to cold-contracture which sets in as a muscle is cooled under conditions of moderate to high pH and ATP level, rigor shortening begins only as the ATP starts to fall sharply during the fast phase of rigor and is always succeeded immediately by rigor itself (see fig. 1). Unlike cold-contracture, it is irreversible. As we have said, it seems to occur whenever the ATP level in a sarcomere falls below about 0.1 p.mole g^{-1} (White, 1970). Although it can develop quite high tensions in excess of 120 gcm^{-2}, it can do only small amounts of work, and is, moreover, highly temperature dependent in the reverse sense to cold-contracture. Thus the work done at 18° during rigor shortening of rabbit psoas is only 0.1 $mJ.g^{-1}$, rising to a maximum of 0.6 $mJ.g^{-1}$ at 38° (Bendall, 1960). This compares with the maximal work done during cold contracture of beef diaphragm muscle (see later) of 2.2 $mJ.g^{-1}$.

Strangely enough, a long-drawn out contracture, akin to rigor shortening at higher temperatures, occurs in beef and pig muscles at 2° or below and can be quite clearly distinguished from the cold-contracture phase proper, either in tension experiments (Lacourt, 1972) or under isotonic conditions, when the load on the muscle is chosen correctly. The best examples of the two phases of cold-shortening are obtained with muscles of high initial pH, because then the two phases do not run into one another. As Lacourt's (1972) results clearly show, tension builds up during the initial cold-contracture phase as soon as the temperature falls below 11°, but this tension rapidly falls again soon after reaching a maximum, i.e. the muscle relaxes spontaneously. Tension then redevelops slowly after a short delay, and follows the time course to be expected if this phase, like rigor shortening at higher temperatures, were dependent on the falling level of ATP (see figs. 3 and 4). It can develop considerably more tension than the cold-contracture phase proper. The second shortening phase was overlooked by earlier investigators (Locker and Hagyard, 1963; Marsh and Leet, 1966), probably because they usually studied free shortening, where the cold-contracture phase encounters no resistance from a load and can therefore become so extensive that further shortening during rigor is prevented. In the practical handling of meat, second phase shortening is probably of more importance than cold-contracture, because the musculature on a rapidly cooled carcass is in fact under varying degrees of tension and not able to shorten freely. For these reasons, we shall describe the phenomenon in some detail.

III Pattern of rigor during cold-contracture

Unlike the experiments of Lacourt (1972), most of ours were carried out under isotonic conditions, i.e. shortening under loads varying from zero to nearly the full isometric tension the contracture can develop at 2°. The muscle strips of about 0.5 cm^2 in cross-section, and 0.8 cm in diameter, were submerged in a bath of H_2O-saturated paraffin (density 0.8) with streams of N_2 bubbling over them. The strips were pre-loaded with 7 - 10 gcm^{-2} and varying afterloads subsequently added in such a way that the extra load was just supported on the bottom of the bath until the strip developed enough tension to lift it. The following beef muscles have been examined in detail: LD, TFL, STM AND D. Simultaneous measurements of pH, PC and ATP were made in separate strips of the same dimensions as the above.

The two phases of shortening and their dependence on load can be demonstrated by comparing the shortening by strips of the same muscle under varying loads (see fig. 3, STM and fig. 4, D). In fig. 3 the shortening pattern of an STM muscle strip under zero load is compared with that of a strip under a load of 39 gcm^{-2}, which in this muscle is near optimal for maximum work performance in phase 1. We see that strip A under zero load shortens rapidly, from the moment it is immersed in the 2° bath, by about 32% of l_o ($\frac{3}{4}$ hr) and then very slowly for the next 23 hr (final shortening 42%). The loaded strip, on the other hand, initially shortens more slowly (by 25% l_o); shortening then abruptly ceases and relaxation sets in, the muscle lengthening under the load back to 95% l_o (2.5 hr). At 3 hr a second slow phase of shortening sets in and continues for the next 21 hr at a low rate, the final shortening being 42% l_o, identical with the shortening of the unloaded strip. The initial shortening/relaxation phase seems to take place while the ATP level (top half of figure) remains nearly constant and high, whereas the second shortening phase coincides with the slow loss of ATP from about 3 hrs onward. By the time the ATP level has fallen by about 1 µ.mole g^{-1}, second phase shortening has overtaken that in the first phase (7 hrs). From 7 to 24 hr shortening continues from 27 to 42% l_o, while the ATP level falls by another 3 µ.mole g^{-1}. The pH has dropped meanwhile from 7 to 6.5 (at 7 hr) and to 6.02 (24 hr - not shown in figure). Another strip of STM was loaded in this experiment with 62 gcm^{-2}, but failed to lift the load in the first phase, and shortened by less than 1% l_o in the second phase.

The experiment with a D muscle in fig. 4 differs in several respects from that with the STM muscle. First, D muscles can lift far greater loads than STM muscles ($>$ 210 gcm^{-2}); secondly all 4 strips in fig. 4 show two phases of shortening quite clearly, even strip A which was loaded with only 7 gcm^{-2}; lastly, second phase shortening comes to an abrupt end about 5 hr

SHORTENING (% l_o) PC OR 2XATP (µMOLES g.$^{-1}$)

FIG. 3

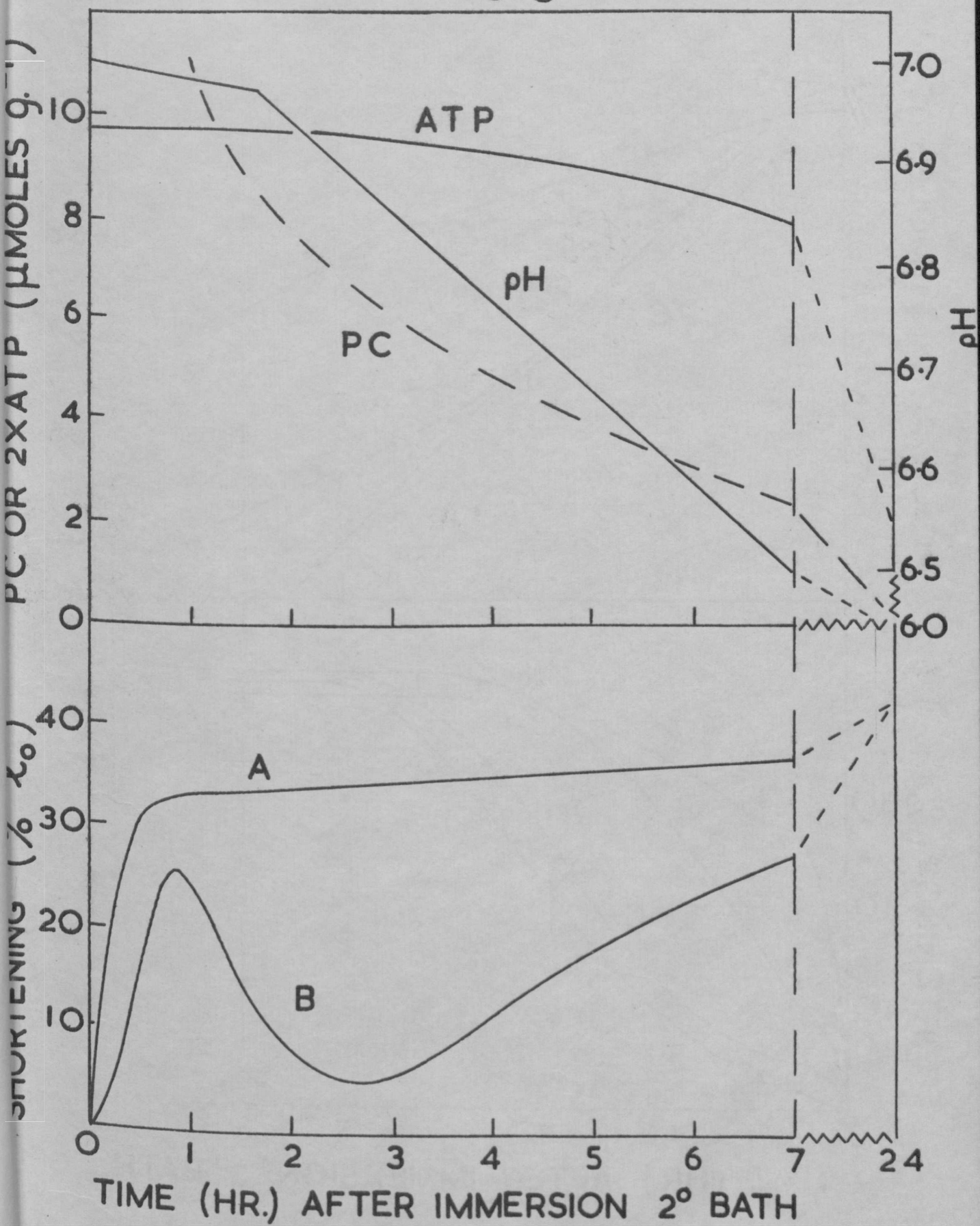

Fig. 3. Pattern of changes in strips of beef STM muscle at 2°. Strip A under zero load; strip B loaded with 39 gcm^{-2}.

FIG.4

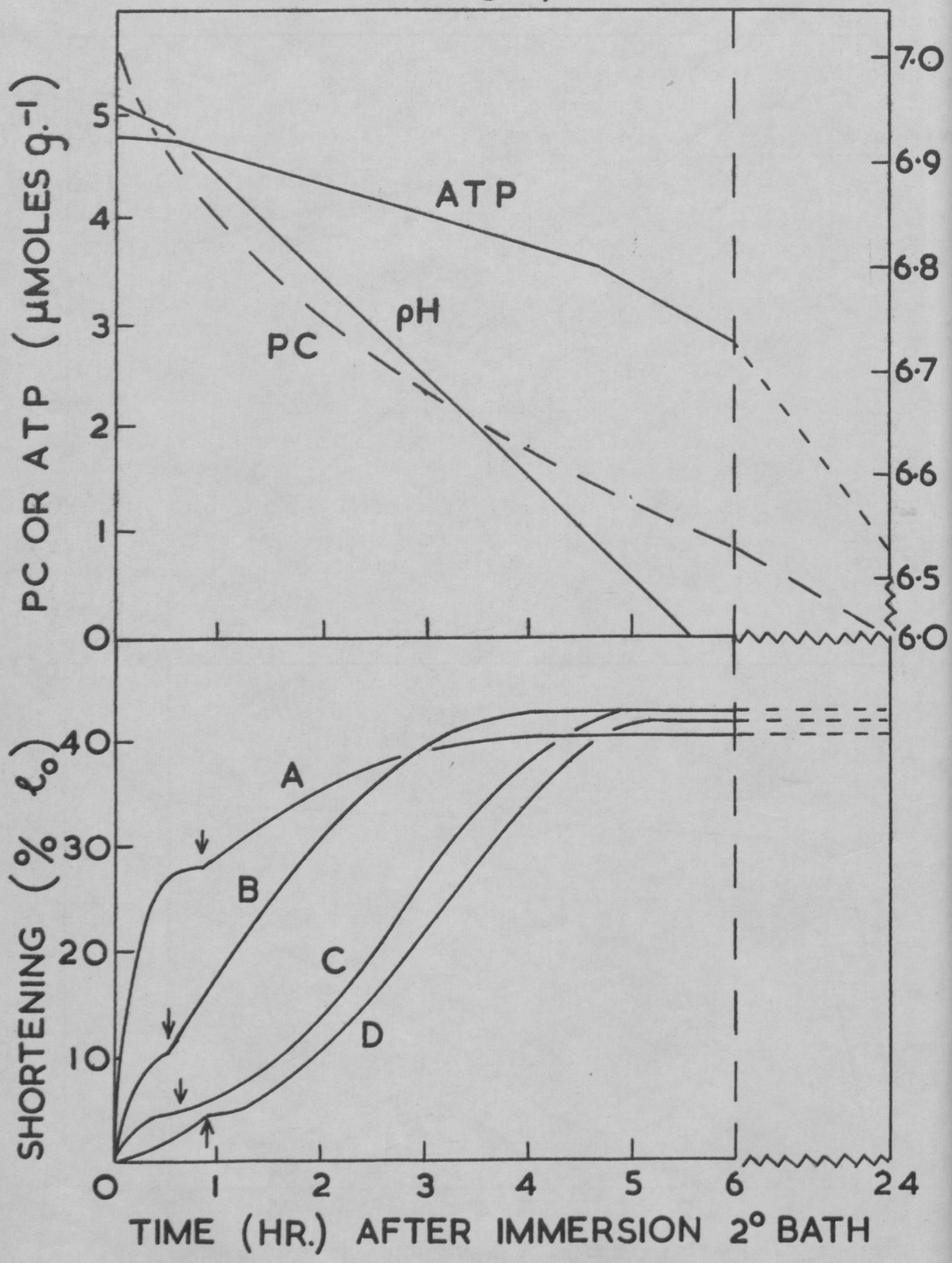

Fig. 4. Pattern of changes in strips of beef D muscle at 2°.
Strips loaded with; A 7; B 86; C 150; D 210 gcm^{-2}.
Start of phase 2 shortening shown by arrows.

after immersion. The effect of load on speed of shortening in phase 1 is very obvious in this experiment; also we see how the higher loads restrict phase 1 shortening drastically, whereas they have no effect on phase 2, where all the muscles finally shorten by 40 to 43% l_o. During second phase shortening, the ATP level falls here by about 1.2 µ.mole g^{-1} and the pH from 6.95 to 6.5.

To prevent all shortening in D muscles loads in excess of 250 gcm^{-2} are needed, compared with only about 60 in STM muscles and less than 40 gcm^{-2} in TFL muscles. LD muscles behave similarly to D muscles in some respects, but cannot lift such heavy loads (maximum = 120 gcm^{-2}). Like STM, they frequently show a relaxation phase at the end of phase 1, whereas D muscles generally do not. It is puzzling that the TFL of beef animals is so easily prevented from cold shortening; indeed it rarely shortens in phase 1 by more than 10% l_o, even under zero load, though the second phase can be quite extensive, but only at very low loads, where it can reach 35% l_o. This contrasts with Lacourt's (1972) results with veal TFL which appears to be the most energetic of the muscles he studied. A possible reason is the low mean initial pH of TFL ($<$ 6.8), although initial pH values in this range have little or no effect on shortening in either D or LD muscles.

Another way of expressing the relative strengths of shortening in the two phases is to plot the work done against the load on the muscle. This is shown in fig. 5 (graph a - phase 1; graph b - phase 2), where we see that D is by far the most energetic of the muscles, doing a maximum of 2.2 $mJ.g^{-1}$ during the first phase at a load of 150 gcm^{-2}, whereas LD can do only about 1 mJ (at 60 gcm^{-2}), STM about 0.7 mJ (at 22 gcm^{-2}) and TFL about 0.1 mJ (at 10 gcm^{-2}). Above these optimal loads, the work done falls rapidly, becoming zero at loads the muscle is just unable to lift, i.e. when the load is just equal to the isometric tension the muscle can develop. This point is at loads of about 260, 130, 60 and 20, respectively, for D, LD, STM and TFL.

Second phase shortening (fig. 5b) does more work than phase 1 in all 4 muscles, except at very low loads, where so much shortening occurs in phase 1 that phase 2 is limited (in general, it seems that overall shortening is restricted to about 45% l_o). In D muscles, for example, phase 2 work, at a load of 150 gcm^{-2}, is 1.4x that in phase 1, and at a load of 210 gcm^{-2} it is more than 5x. LD muscles behave similarly. Though not as energetic as D muscles, they can perform a maximum of 3.4 $mJ.g^{-1}$ at optimal loads, of which 3 $mJ.g^{-1}$ is done in the second phase which we have likened to rigor shortening at higher temperatures, The latter, however, cannot do as much work; for example, Marsh (1954) found that LD muscles shortening under load during rigor at 38° performed a maximum of only 0.75 $mJ.g^{-1}$.

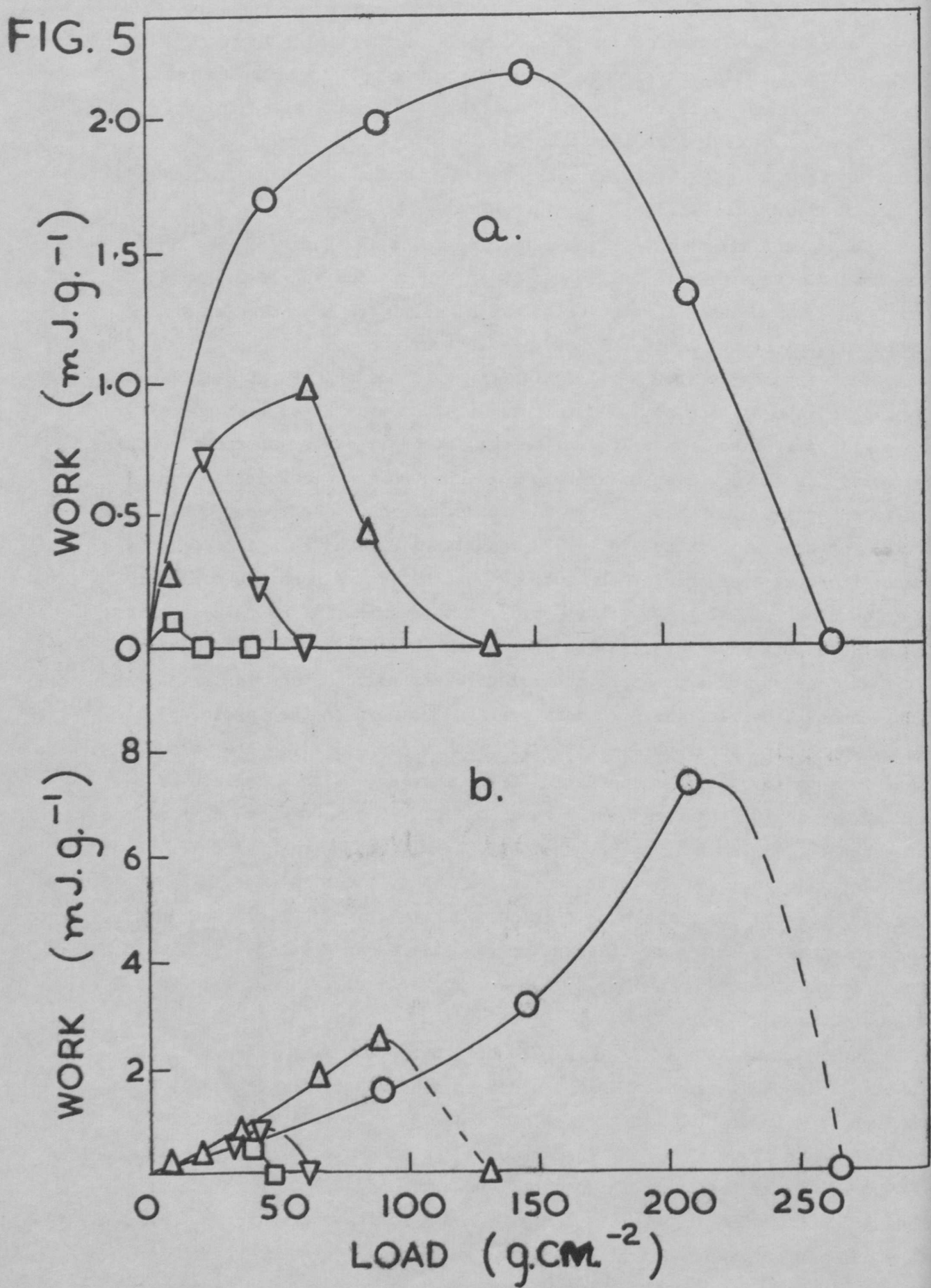

Fig. 5. Work done by muscles, shortening at 2°: A, work done in cold-contracture (phase I); B, work done in rigor-shortening (phase II)

○ = D; △ = LD: ▽ - STM: □ = TFL

The effect of load on the speed and extent of phase 1 shortening is similar to that which it has in living contractions of frog muscle, for example, (Hill, 1965; cf. Bendall, 1969), lowering the speed in an approximately exponential manner. Both speed and extent become zero, of course, when the load on the muscle ≡ the isometric tension which can be developed. In this respect phase 2 shortening differs markedly, as fig. 4 demonstrates. Providing phase 1 shortening has not been too extensive, the speed of shortening in phase 2 is far less dependent on load than that of phase 1. In fact, in the case of the D muscle in fig. 4, the speed of second phase shortening was almost identical at loads of 86, 150 and 210 gcm^{-2} (curves B, C and D respectively). Like the first phase, however, phase 2 shortening is abruptly and completely inhibited as the load gets close to the isometric tension (about 260 gcm^{-2}, in this case). Phase 2 also differs from phase 1 in that it appears to be quite independent of pH. In one case of a TFL muscle, for example, a load of 40 gcm^{-2} completely prevented phase 1 shortening, and phase 2 did not set in until 7 hr after immersion in the bath. By this time the muscle pH was below 6.3.

Some of the important factors involved in cold-contracture are set out in table III.

Table III. Important parameters in cold-contracture in beef muscles at 2°

Muscle	V_A [a]	V_{pH} [b]	V_S [c]	Max. work ($mJ.g^{-1}$) in phase 1	Max. work ($mJ.g^{-1}$) in phase 2	pH 0	pH 23	pH 47 hr	ATP μ.mole g^{-1} 23	ATP μ.mole g^{-1} 47 hr
LD	8.5	.09	.34	1.0	2.5	6.80	5.67	5.58	0.3	<.2
TFL	7.0	.07	0	0.1	0.5	6.78	5.78	5.66	1.5	<.3
STM	8.9	.09	.03	0.7	0.8	7.12	6.04	5.77	1.0	<.3
D	8.8	.10	.63	2.2	7.5	6.85	6.02	5.80	0.3	<.2

a. V_A = rigor ATP-turnover rate in μ.mole $hr^{-1} g^{-1}$

b. V_{pH} = rate of pH fall in units hr^{-1} (6.85 - 6.2)

c. V_S = speed of phase 1 shortening in muscle lengths hr^{-1}

We note that the ATP-turnover rate and rate of pH fall are remarkably similar from muscle to muscle, whereas the shortening rates and the maximal work which can be done in the two phases of shortening differ widely. It is noticeable that neither the ultimate pH nor the ultimate ATP level is reached after 23 hr immersion at 2°. In TFL and STM muscles, indeed, $\frac{1}{5}$ of the initial ATP is still left at 23 hr. Note that higher initial pH values than the mean ones shown here can occur in LD, TFL and D muscles, but they are rare.

THE RELATION OF COLD-CONTRACTURE TO MAXIMAL CONTRACTION SPEED AND TO REDNESS

The interrelation of redness (= myoglobin content), speed of contraction and ability to cold shorten is an extremely vexed one which cannot be discussed fully here. Nevertheless the present results, like those of Lacourt (1972), point up some of the anomalies. As we shall see, it is true in extreme cases that the reddest muscles of an animal contract slower, but cold-shorten faster and more extensively than the whitest ones, though the relationship is far from strict. Our own limited results with beef and pig muscles are summarised in table IV, where we have used the maximal, Ca-stimulated, MgATP-actin-myosin ATP-ase activity (V_m) of myofibrils as the criterion of contraction speed. This activity was assayed with 4 mM $MgATP^{2-}$ as substrate, and was stimulated with 0.1 mM Ca^{2+} at a pH of 7.2 and I = 0.15. The activities have been corrected to μ.mole ATP split $hr^{-1}g^{-1}$ (fresh muscle). Rabbit psoas is included for comparison.

Table IV. Comparison of myoglobin content, contraction speed and ATP-turnover rate with speed of cold-contracture in muscles of beef, pig and rabbit.

Muscle	V_{S_1} [b] 2°	Load gcm^{-2}	M content nmole per g	V_m [c] 35°	V_m [c] 2°	V_A [d] 2°
Beef LD	0.34	50	280	9400	105	8.5
TFL	0	50	265	7700	86	7.0
STN	0.03	50	250	5800	65	8.9
D	0.63	50	420	2700	30	8.8
Pig[a] VL(R)	1.51	0	190	3200	36	-
VL(W)	0.36	0	45	8000	89	-
BF(R)	1.05	0	100	6800	76	-
BF(W)	0.20	0	41	7600	84	-
Rabbit[a] PS	0	0-50	10	14300	160	9.0
ST[a]	0.25	3	200	3000	33	-

a. VL = vastus lateralis; BF = biceps femoris; ST = semitendinosus; R = red portion; W = white portion

b. V_{S_1} = speed of cold contracture (phase 1) in muscle lengths per hr.

c. V_m = max. ATP-ase activity in μ.mole $hr^{-1}g^{-1}$. This varies directly with contraction speed.

d. V_A = observed ATP-turnover rate during cold-contracture in μ.mole $hr^{-1}g^{-1}$.

The table shows that in beef muscles the relation between redness (myoglobin content) and either speed of contraction (V_m) or of cold contracture (V_{S_1}) is tenuous to say the least. Only in the extreme case of beef D are the values for myoglobin content and speed of contracture in agreement and these are related to an extremely low contraction speed, so that here we have a classical case of a slow, red muscle which cold-contracts vigourously. On the other hand, the LD muscle disobeys this rule completely, because it has the highest contraction speed of all, is not particularly red and yet also shows a high rate of cold-contracture. The other two beef muscles, TFL and STM, show intermediate contraction speeds, yet have similar myoglobin contents to the LD, but are only capable of quite feeble cold-contracture.

Similar contradictions exist amongst the pig muscles where the red portions of VL and BF show quite vigourous cold-contracture, yet BF(R) has more than double the contraction speed of VL(R), and is also less red. The white portions of these muscles show only feeble phase 1 cold-contracture, although they are capable of shortening considerably in phase 2 (rigor). Both white portions have high contraction speeds. On the other hand, the two rabbit muscles behave classically; the fast, white PS shows no cold-contracture at 2°, whereas the very slow, red semitendinosus cold-contracts quite fast.

One can only conclude that the red-slow/white-fast rule applies only in the most extreme cases of very red and very white muscles, the former showing vigourous cold-contracture and the latter no cold-contracture at all. It has been suggested (Horgen et al., 1972) that very slow red muscles show cold-contracture because they have only very sparse sarcoplasmic reticula, and hence very inefficient Ca-pumps which will allow extensive Ca-release at low temperatures (Bendall, 1973), whereas very fast, white muscles have the very opposite. This may be true in some extreme cases, but it does not apply to either the beef or pig muscles studied here. If it were true then the redder muscles, allowing escape of more Ca^{2+} at low temperature, would be expected to have higher ATP-turnover rates (V_A) during rigor than the whiter, whereas table IV shows that V_A is remarkably similar from muscle to muscle in beef animals. In contrast, rabbit PS, which is very fast and very white, should have the most efficient Ca-pump of all, yet in fact it shows nearly the same ATP-turnover rate during low temperature rigor as beef D does.

The rigor turnover rates of ATP are, indeed, somewhat of a thorn in the side of the Ca-release hypothesis, because they are not only similar amongst muscles which show quite different speeds of cold-contracture, but they are also nearly constant throughout the whole rigor-process in any given muscle.

This, in spite of the fact that both beef LD and STM muscles, for example, show quite definite relaxation phases at the end of phase 1; during such relaxation one would expect V_A to fall due to reduction of the intrafibrillar Ca^{2+} level, but nothing of the sort can be detected, at least by present analytical methods. No doubt these contradictions will be resolved by further research.

THE IMPORTANCE OF RIGOR SHORTENING AND COLD CONTRACTURE IN THE PRACTICAL HANDLING OF MEAT.

The practical importance of muscle shortening during all types of rigor cannot be overstressed, as the New Zealand workers have repeatably shown (Locker and Hagyard, 1963; Marsh and Leet, 1966; Davey and Gilbert, 1969), because of its effect on the toughness of meat. It is the degree of shortening which brings about toughening, and not the means by which shortening is achieved; the results of Herring et al. (1965) on the toughness of various muscles of normally treated beef carcasses, hung up in the traditional manner by their hind-limbs, amply confirm this conclusion. They found that those muscles which had been able to shorten during rigor because of the carcass posture, notably those on the back of the thigh, were all tougher than the relatively stretched muscles, such as those on the front of the thigh. By altering the posture of the carcass during rigor, by lying it on its side with the legs flexed, the relative shortening was reversed and with it the toughness, muscles on the back of the thigh then being more tender than those on the front. Incidentally, these findings should be a warning to any butcher who thinks it a good idea to bone out meat while it is still hot. Extensive rigor shortening will occur in the meat in this state, with resulting toughening post-rigor.

In the above experiments there was no question of cold-contracture, but merely the effect of posture, so that the flexed muscles were found to have nearly uniform, but 'short' sarcomere lengths and the extended muscles 'long' sarcomere lengths. When cold-contracture, due to very rapid chilling of the carcass, is superimposed on the posture effect, both flexed and extended muscles tend to be tougher than normal. This is because, even in an extended muscle, some sarcomeres shorten quicker than others due to unequal rates of cooling, and thus shortened nodes, alternating with lengthened portions, tend to form throughout the musculature (cf. the model experiment of Marsh and Leet (1966) to demonstrate this point). At MRI, we have shown how unequally sarcomeres within adjacent fibres do in fact shorten during cold-contracture, even when the muscle is restrained from any overall

shortening (Voyle, 1969; Restall and Voyle, unpublished observations). Longitudinal sections, examined over at least 2 mm length of muscle, show every conceivable pattern of shortening, including node formation, passive crimpling and extended sarcomeres. Even in rigor at 15°, we have noted considerable variation in sarcomere length in restrained muscles, but not to the same extent as in cold-contracture.

The occurrence of phase 2 shortening after phase 1 cold-contracture is complete, adds a further hazard to any attempt to cool carcasses very rapidly, because it greatly extends the dangerous period during which the muscle temperature should not be allowed to drop below 10°, if shortening and consequent toughening are to be avoided. In beef carcasses, this may not be a serious problem, because there is such a mass of musculature that rapid cooling is likely to occur only in a superficial layer, but it is certainly hazardous in calf or lamb carcasses and possibly in pig. The results presented here strongly suggest that all types of carcass would be optimally tender if they were hung at 12° for at least 15 hr to allow the completion of rigor, before any attempt was made to cool them below 10°. After 15 hr, it is safe to chill as rapidly as practicable (cf. Bendall, 1971).

There is, at present, no satisfactory theoretical explanation of why cold-shortened muscles should be tougher than muscles at rest length. The question is bedevilled by the fact that muscle lengths, and sometimes sarcomere lengths, have usually been measured on the uncooked meat, whereas the toughness measurements have been made after cooking. Quite apart from other objections, this method fails to take into account the more or less severe cooking shrink the muscles undergo, due to heat shrinkage of their collagen. Furthermore, the denaturation and coagulation which occurs during cooking causes extensive cross-linking of the denatured proteins into confirmations quite different from those in the fresh material.

Uncooked, cold-shortened muscle is in fact not much tougher and often more tender than unshortened muscle (Drs. E. Dransfield and D. N. Rhodes, MRI - unpublished observations). This is expectable on the sliding filament hypothesis, because when a fibre has shortened beyond 35% of its rest length its constituent actin filaments overlap each other and, moreover, its myosin filaments become distorted by pulling themselves up against the Z-discs (see fig. 1). The result is that the number of rigor cross-links which can be formed between actin and myosin filaments is vastly reduced, and consequently the resistance of the fibre to stretch or compression falls.

This effect has its reflection in tension experiments with living muscle, where the active tension falls to zero when the sarcomere length is reduced to the point at which myosin filaments begin to bang into the Z-discs, thereby drastically reducing the contractile links which can form between myosin heads and actin monomers (Gordon et al., 1966).

The situation is probably very different after cooking, because the highly distorted sarcomeres in the shortened nodes are in an ideal state to form tough, compact coagula as the muscle proteins are denatured. It is noteworthy, indeed, that in very highly shortened regions of the muscle, the sarcomere lengths are often less than the length of a myosin filament (i.e <1.6 μm), indicating that more than 50% shortening has occurred locally, in spite of the overall shortening being perhaps less than 40% of the initial muscle length. At present this seems the best explanation of the toughening which accompanies shortening, but it is far from adequate.

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SUMMARY

1. The fundamental biochemical and physical background of rigor mortis is discussed in detail. It is shown that the physical basis of the loss of stretchability during rigor is the formation of cross-links between the heads of the molecules in the myosin filaments and the monomers which make up the actin filament. This occurs as the ATP level falls from its resting level of 5 - 8 μ.mole g^{-1} to < 0.1 μ.mole g^{-1}. Loss of the last traces of ATP causes a localised contraction, accounting for rigor shortening.

2. The biochemical events are dominated by the slow turnover of $MgATP^{2-}$ at the ATP-ase sites of the myosin heads. This turnover is counterbalanced at first by resynthesis of ATP from PC and glycolysis (lactate formation; fall of pH), until the PC supply runs out, when the ATP level falls, due to splitting to IMP, NH_4^{1+} and P_i.

3. Temperature has anomalous effects on the rate of turnover of ATP, so that the rate falls fast at first and then ever more slowly as the temperature is reduced below 15°. In beef muscles the rate at 2° is as high as at 15°, thus foreshortening the rigor process in a manner not predictable from simple thermodynamic theory.

4. Cold-contracture accompanies the anomalous biochemical behaviour at low temperatures. It is thought to be due to release of Ca^{2+} ions from their storehouse in the SR, this release stimulating the contractile actin-myosin ATP-ase, previously dormant.

5. The extent of cold-contracture varies inversely with the load on the muscle, and so does its speed. Its duration is confined to about the first ¾ hr after cooling to 2°. It is sometimes followed by a relaxation phase which is in turn supplanted by further slow contraction (phase 2 rigor shortening). The latter often exceeds in extent the cold-contracture and can perform more work. Phase 2 is not highly load or pH-dependent.

6. The bearing of these changes on the practical handling of meat is discussed, particularly in relation to the toughening which accompanies extensive shortening of the muscle.

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