Post-Mortem Glycolysis in Ox Skeletal Muscle

EFFECT OF PRE-RIGOR FREEZING AND THAWING ON THE INTERMEDIARY METABOLISM

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1. Ox sternomandibularis muscle was 'slow-frozen' by placing it in air at -22° or 'fast-frozen' by immersion in liquid air or acetone-solid carbon dioxide. In all cases muscles were frozen pre-rigor. Changes in length, pH and the concentrations of P_i , creatine phosphate, hexose monophosphate (glucose 1-phosphate + glucose 6-phosphate + fructose 6-phosphate), fructose diphosphate (fructose 1,6-diphosphate + $\frac{1}{2}$ triose phosphate), lactate, ATP, ADP, AMP and NAD^+ during freezing and during subsequent thawing were determined. In addition some measurements were made of the changes in α -glycerophosphate, 3-phosphoglycerate, 2-phosphoglycerate, phosphoenolpyruvate and pyruvate concentrations during slow freezing. 2. Appreciable shortening and marked changes in chemical composition took place during slow freezing but not during fast freezing. 3. During slow freezing the hexose monophosphate concentration fell and fructose 1,6-diphosphate and triose phosphate increased substantially. Increases also took place in 3-phosphoglycerate, 2-phosphoglycerate and phosphoenolpyruvate, but not in pyruvate. 4. On thawing, most of the chemical changes were similar to those in unfrozen muscle *post mortem*, but took place much more rapidly; loss of NAD^+ was particularly rapid. Fast-frozen muscle metabolized at a faster rate on thawing than did slow-frozen muscle. 5. The overall changes in length during freezing and thawing were about the same in slow-frozen as in fast-frozen muscle.

Thaw rigor, a phenomenon that occurs when muscle frozen in a pre-rigor state is thawed, is characterized by extreme shortening and very rapid loss of ATP and creatine phosphate (Bendall, 1960). The present paper reports the results of an investigation of the changes occurring in the concentrations of several glycolytic intermediates and cofactors during the thaw rigor process. Early in this investigation it was found that muscle frozen pre-rigor by placing it at -22° differed significantly from unfrozen muscle with respect to the concentrations of certain constituents. Hence the changes occurring during freezing at -22° , and during rapid freezing in liquid air or in acetone-solid carbon dioxide, were also investigated. The results of a similar study of the changes occurring during the development of rigor mortis in unfrozen muscle were reported in a previous paper (Newbold & Scopes, 1967).

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MATERIALS AND METHODS

Ox sternomandibularis muscles were obtained as described previously (Newbold & Scopes, 1967). Strips (about 0.5 cm.² in cross-section and 3 cm. long) in which the fibres ran longitudinally were dissected from them and frozen, about 1.5 hr. *post mortem*, by the following methods. The strips were (a) dropped into liquid air, (b) dropped into acetone-solid CO_2 after first sealing them in polythene, or (c) placed on thin polythene sheeting in Petri dishes at room temperature ($20-23^{\circ}$) and the covered dishes were then placed in air at -22° . This last method of freezing is referred to as slow freezing. Thawing was effected by removing the strips from frozen storage and placing them in air at room temperature on glass plates covered by thin polythene sheeting.

Methods of pH measurement and of preparation and analysis of perchloric acid extracts were as described previously (Newbold & Scopes, 1967). Hexose monophosphate values are the total of glucose 1-phosphate, glucose 6-phosphate and fructose 6-phosphate, whose concentrations were measured by adding all the relevant enzymes simultaneously. In only one experiment were the

Table 1. *Differences in pH and chemical composition between muscles slowly frozen pre-rigor and stored at -22° for 4 hr. or 24 hr. and the corresponding muscles at zero time*

Experimental details are given in the text. The results are given as mean differences ($\pm$ S.E.M.) from the initial unfrozen samples. The numbers of muscles are shown in parentheses.

Time Change in pH Compound	Change in concn. (μ moles/g.)	
	4 hr.	24 hr.
	-0.14 ± 0.03 (7)	-0.20 ± 0.04 (7)
Lactate	$+6.6 \pm 2.0$ (8)	$+8.6 \pm 1.8$ (7)
P _i	0 ± 0.9 (8)	$+0.9 \pm 1.2$ (7)
Creatine phosphate	-2.6 ± 0.8 (8)	-3.7 ± 0.8 (7)
Hexose monophosphate	-2.23 ± 0.40 (8)	-1.81 ± 0.20 (7)
Fructose diphosphate	$+2.44 \pm 0.15$ (8)	$+2.67 \pm 0.20$ (7)
ATP	-0.12 ± 0.15 (8)	-0.37 ± 0.20 (6)
ADP	$+0.03 \pm 0.06$ (8)	-0.07 ± 0.07 (7)
AMP	$+0.11 \pm 0.03$ (8)	$+0.10 \pm 0.03$ (7)
NAD ⁺	$+0.02 \pm 0.01$ (6)	-0.01 ± 0.01 (5)

fructose 1,6-diphosphate and triose phosphate (glyceraldehyde phosphate + dihydroxyacetone phosphate) concentrations measured separately. In the others, by simultaneous addition of the relevant enzymes, the sum of the fructose 1,6-diphosphate plus half the triose phosphate was measured. This sum is referred to as fructose diphosphate.

RESULTS

The overall changes after 4 hr. and 24 hr. of the slow freezing conditions are summarized in Table 1. Zero-time samples were taken at the time the strips were exposed to freezing conditions. Mean differences only are given in Table 1 because of the wide variation in initial values between muscles (Newbold & Scopes, 1967). The principal changes apart from the loss of creatine phosphate and the decline in pH (which would be expected without freezing) were the decrease in hexose monophosphate and the increase in fructose diphosphate. Since the fructose diphosphate concentration in the unfrozen muscle strips was not more than 0.05μ mole/g. (cf. Newbold & Scopes, 1967), the change of $+2.44 \mu$ moles/g. represents a considerable increase. AMP also increased markedly; however, the initial AMP concentrations were considerably lower in these muscles than in those examined previously (Newbold & Scopes, 1967). Freezing with acetone-solid carbon dioxide (three experiments) or liquid air (two experiments) caused no marked change in any constituent. The frozen samples were all extracted in the same way, by dropping them into cold perchloric acid and homogenizing immediately. Since the analyses of the fast-frozen samples did not differ significantly from those of the zero-time (unfrozen) samples, it is clear that the differences between the unfrozen and slow-frozen muscle strips were not due to the methods of extraction.

Progressive changes during slow freezing were investigated by placing a number of strips at -22° and removing them at intervals for analysis. The rate of cooling was followed by means of a thermocouple inserted at the centre of one of the strips. The results obtained (means for four muscles) are given in Table 2 and Fig. 1.

Freezing commenced after about 12–15 min., after some supercooling (on one occasion to -9°). The pH and the creatine phosphate concentration decreased rapidly up to the time freezing commenced, and thereafter at a much lower rate. The fructose diphosphate concentration began to increase at about the time freezing commenced. The hexose monophosphate concentration increased slightly during the pre-freezing period but then decreased by amounts closely paralleling the increases in fructose diphosphate. ATP, ADP and NAD⁺ concentrations changed little throughout the entire period, but the AMP concentration increased during the first hour. Lactate concentrations, though they fluctuated widely (because of large differences among the strips at zero time), showed a tendency to increase with time, the increase being most rapid during the cooling phase, but continuing slowly after the temperature had fallen below -20° . The P_i concentration did not change significantly throughout the entire period.

In addition to the compounds mentioned above the C₃ compounds of glycolysis (except 1,3-diphosphoglycerate) were measured. In one experiment triose phosphate and fructose 1,6-diphosphate concentrations were measured separately. They were 0.03 and 0.04μ mole/g. respectively at zero time. In six muscle strips that had been held at -22° for 4 hr., 24 hr. or 48 hr. they were appreciably higher and averaged 0.58 and 1.92μ moles/g.

Table 2. pH and concentrations of compounds in muscle slowly frozen pre-rigor and stored at -22° for various times

Experimental details are given in the text. All values are the means for the same four muscles (pH values at zero time, 6.63–6.77).

Time at -22° pH Compound	Concn. (μ moles/g.)			
	0 hr. 6.69	1 hr. 6.57	4 hr. 6.51	24 hr. 6.45
Lactate	29.7	36.5	35.2	39.4
P _i	18.4	19.0	19.3	20.6
Creatine phosphate	3.7	2.1	1.3	1.4
Hexose monophosphate	4.52	3.08	2.18	2.50
Fructose diphosphate	0.03	2.06	2.60	2.74
ATP	4.51	4.48	4.30	4.16
ADP	1.26	1.18	1.26	1.14
AMP	0.11	0.28	0.31	0.26
NAD ⁺	0.38	0.39	0.39	0.37

Fig. 1. Changes in (a) internal temperature and (b) pH (●) and the concentrations of creatine phosphate (○), hexose monophosphate (□) and fructose diphosphate (■) in pre-rigor muscle strips during the first 60 min. after they were placed at -22° . Experimental details are given in the text. Points are mean values for four muscles.

respectively. The results of three experiments in which the concentrations of 3-phosphoglycerate, 2-phosphoglycerate, phosphoenolpyruvate, pyru-

vate and α -glycerophosphate were measured are summarized in Table 3. The concentrations of the first three of these compounds increased during freezing, but the pyruvate concentration did not change significantly.

Effect of thawing

In these experiments muscle strips were removed from frozen storage and allowed to thaw and shorten in air at room temperature. At intervals the pH was measured and perchloric acid extracts were made. Length changes in several strips were also recorded.

Measurements were made on seven sets of strips, one set from muscle 1 and two sets each from muscles 2, 3 and 4. One set from muscle 3 was fast-frozen in acetone-solid carbon dioxide and one set from muscle 4 in liquid air. The remainder were slow-frozen. All strips were free to shorten during freezing except for those of one of the sets from muscle 2, which were prevented from shortening by clamping them at both ends before freezing. These strips were cut free from the clamps on removal from frozen storage. Both sets from muscle 2 were thawed at 96 hr. and all other sets at 24 hr. after freezing.

Slow-frozen muscle. The averaged results for the four slow-frozen sets, which were free to shorten during freezing, are presented in Figs. 2–5. During the first 10 min. little change occurred except for some decrease in creatine phosphate, a small increase in hexose monophosphate and a small decrease in fructose diphosphate. After the ice had melted the strips were able to shorten; Fig. 2 shows that rapid shortening commenced at about 10 min. Shortening was 80% complete by about 20 min. and complete by 40 min., when the strips had shortened, on average, to 40% of their frozen length. The

Table 3. Concentrations of some glycolytic intermediates in muscle at zero time and after slow freezing

Experimental details are given in the text. All results are the means for the same three muscles, strips of which were stored at -22° for periods ranging from 1 hr. to 96 hr.

Compound	Concn. (μ moles/g.)		
	Unfrozen muscle (at zero time)	Frozen muscle	Increase during freezing ($\pm$ S.E.M.)
Fructose 1,6-diphosphate + $\frac{1}{2}$ triose phosphate	0.05	2.49	2.44 ± 0.12
α -Glycerophosphate	2.76	2.90	0.14 ± 0.09
3-Phosphoglycerate	0.04	0.36	0.32 ± 0.05
2-Phosphoglycerate	0.02	0.14	0.12 ± 0.01
Phosphoenolpyruvate	0.02	0.08	0.06 ± 0.01
Pyruvate	0.13	0.15	0.02 ± 0.01

Fig. 2. Time-course of the changes in length (○) and pH (●) that occurred when muscle frozen pre-rigor was thawed. Experimental details are given in the text. Points are mean values for four muscles.

Fig. 3. Time-course of changes in the concentrations of ATP (○), creatine phosphate (●) and P_i (Δ) that occurred when muscle frozen pre-rigor was thawed. Experimental details are given in the text. Points are mean values for the same four muscles as in Fig. 2.

relationship between the decrease in pH (Fig. 2) and the increase in the concentration of lactate was equivalent to about $60 \mu\text{moles/g.}/\text{pH}$ unit (cf. Newbold & Scopes, 1967). In all sets the ultimate pH was reached by 150 min.

A considerable amount of thaw shortening took place before there was any significant decrease in ATP concentration (cf. Bendall, 1960) or in pH. Changes in ATP, creatine phosphate and P_i concentrations (Fig. 3) were similar to those in unfrozen muscle except for the time-scale. Thus, whereas ATP took about 8 hr. to decrease to half its initial value in intact muscle held under nitrogen at 15° (Newbold & Scopes, 1967), it took only about 30 min. in muscle undergoing thaw rigor (Fig. 3), i.e. it was about 15 times faster. Changes in ADP and AMP concentrations (Fig. 4) were also accelerated by about 15 times. The initial rate at which NAD^+ was lost, however, was accelerated more than this, a fact attributable to release of NAD-hydrolysing enzymes as a result of damage caused to subcellular structures by freezing. In two of the muscles the NAD^+ concentration fell from 0.45 to $0.10 \mu\text{mole/g.}$ by 50 min.

Changes in the hexose phosphate concentrations

Fig. 4. Time-course of changes in the concentrations of ADP (○), AMP (●) and NAD^+ (Δ) that occurred when muscle frozen pre-rigor was thawed. Experimental details are given in the text. Points are mean values for the same four muscles as in Fig. 2.

are shown in Fig. 5. The fructose diphosphate concentration decreased rapidly to less than $0.2 \mu\text{mole/g.}$ In only one of the muscles, where its concentration increased, was there any subsequent

Fig. 5. Time-course of changes in the concentrations of hexose monophosphate (○) and fructose diphosphate (●) that occurred when muscle frozen pre-rigor was thawed. Experimental details are given in the text. Points are mean values for the same four muscles as in Fig. 2.

marked change in concentration. The hexose monophosphate concentration increased rapidly during the period of shortening and then declined, reaching a constant value at about 80 min.

At the time the strips derived from muscle 2 were removed from frozen storage the chemical composition of the 'restrained' strips was very similar to that of the 'unrestrained' strips. Subsequent changes were similar in both sets.

Fast-frozen muscle. Features common to both experiments in which fast-frozen and slow-frozen samples of the same muscle were compared are described below. Mean values are given.

During fast freezing virtually no shortening occurred, whereas during slow freezing the muscle strips shortened to about 65% of their initial length. On thawing, the fast-frozen strips shortened to 28% and the slow-frozen to 40% of their frozen lengths. Thus the total change in length during freezing and thaw rigor was about the same in the fast-frozen as in the slow-frozen sets. As in the slow-frozen muscle shortening started at about 10 min. and was complete by about 40 min.

As indicated in the experiments on the changes occurring during freezing, the chemical composition of the fast-frozen strips was markedly different from that of the slow-frozen ones. However, during thawing, the fast-frozen strips took much longer to warm through the critical sub-zero temperature range than they had taken to cool through this range, and hence it is not surprising that in the first 10 min. changes similar to those occurring during slow-freezing took place. Hexose monophosphate decreased by about $0.8 \mu\text{mole/g.}$ and fructose diphosphate increased by about the same amount. The only other notable change during this period

Fig. 6. Effect of method of pre-rigor freezing on the changes in (a) pH and (b) ATP concentration that occurred when the frozen tissue was thawed: ○, fast-frozen muscle; ●, slow-frozen muscle. Experimental details are given in the text. Points are mean values for two muscles.

was in the creatine phosphate concentration, which decreased somewhat. Subsequent changes in all the compounds measured were essentially the same as in the slow-frozen muscle, but the metabolism was significantly faster. As shown in Fig. 6, the pH and the ATP concentration started to fall at about the same time (15–20 min.) in the fast-frozen as in the slow-frozen sets, but the ATP concentration had fallen to half its initial value and the pH to 6.2 by about 40 min. in the fast-frozen compared with about 50 min. in the slow-frozen sets. The hexose monophosphate peak occurred at about 30 min., as in the slow-frozen muscle, but was sharper owing to a more rapid loss after 30 min. The AMP concentration reached its maximum of $0.53 \mu\text{mole/g.}$ at about 60 min., whereas in the slow-frozen sets the maximum of $0.39 \mu\text{mole/g.}$ was reached at about 80 min. No effect of rate of freezing on the rate of NAD^+ loss during subsequent thawing was apparent.

DISCUSSION

In contrast with the results reported for pig longissimus dorsi (Kastenschmidt, Briskey & Hoekstra, 1966), ox sternomandibularis muscle has

not been found to contain fructose 1,6-diphosphate or triose phosphate in concentrations greater than about $0.05 \mu\text{mole/g.}$ at any time during the development of rigor mortis (Newbold & Scopes, 1967). However, during slow freezing pre-rigor, the concentrations of both these compounds increased very considerably in the ox muscle, indicating that some step(s) subsequent to aldolase became rate-limiting for lactate production. The finding that there were small increases in 3-phosphoglycerate, 2-phosphoglycerate and phosphoenolpyruvate, but not in pyruvate, indicates a block at the pyruvate kinase step. In addition, the proportionately greater increase in triose phosphate than in the C_3 acids suggests that blocks also developed at one or both of the steps mediated by glyceraldehyde phosphate dehydrogenase and phosphoglycerate kinase. The present results do not indicate any lack in the muscle of the other compounds involved in these enzymic steps.

The results relating to the glycolytic metabolism in ox sternomandibularis muscle after thawing confirm and extend the findings of Szentkiralyi (1957) and Bendall (1960), who both used rabbit psoas muscle. Pre-rigor freezing of muscle stimulates the conversion of phosphorylase *b* into the *a* form (Cori, 1956). Though during slow freezing the phosphorylase lagged behind the phosphofructokinase activity (since the hexose monophosphate concentration decreased), immediately after thawing phosphorylase was very active, and a rapid accumulation of hexose monophosphate occurred (Fig. 5). Overall glycolysis was accelerated to many times its rate in unfrozen muscle under nitrogen. However, accelerated glycolysis could not continue without a corresponding acceleration of ATP breakdown, and Bendall (1960) has suggested that the rapid breakdown of ATP during the onset of thaw rigor is due to activation of myofibrillar adenosine triphosphatases by Ca^{2+} liberated by freezing and thawing; Kushmerick & Davies (1968) have presented evidence that is consistent with this view. It would appear likely that the greater adenosine triphosphatase activity in fast-frozen than in slow-frozen muscle on thawing is due to greater Ca^{2+} release in the former.

The activities of phosphorylase and phosphofructokinase, the two key enzymes in the regulation of glycolysis in unfrozen muscle *post mortem* (Newbold & Scopes, 1967), are clearly much greater after freezing and thawing. The possible roles of Ca^{2+} , P_i , adenine mononucleotides and certain glycolytic intermediates in the regulation of the activities of one or both of these enzymes have been

reviewed by Helmreich & Cori (1965) and Stadtman (1966). Comparison of the present results with those obtained for unfrozen muscle did not reveal any differences in total P_i , ATP, ADP, AMP, hexose monophosphate or fructose diphosphate that would account for differences in the rates of these two enzymes. However, as has been discussed by Lowry, Passonneau, Hasselberger & Schulz (1964), local concentrations at the sites of enzymic activity may differ considerably from the total concentrations in the muscle.

If it is assumed that the enzymes creatine kinase and myokinase maintain equilibrium among their reactants, and that the ATP, creatine and creatine phosphate as measured are true indications of the concentrations present at the site of activity of creatine kinase, it can be calculated that over 90% of the ADP and over 99% of the AMP are not freely available in the sarcoplasm during the early stages of glycolysis. Even less is available if part of the ATP is also assumed to be located elsewhere. Thus large changes in the effective concentrations of these controlling factors would not be reflected in the total concentrations as measured by the perchloric acid extraction technique.

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