Post-Mortem Glycolysis in Ox Skeletal Muscle

EFFECT OF TEMPERATURE ON THE CONCENTRATIONS OF GLYCOLYTIC INTERMEDIATES AND COFACTORS

By R. P. NEWBOLD AND R. K. SCOPES*

*Commonwealth Scientific and Industrial Research Organization,
Division of Food Preservation, Ryde, New South Wales, Australia*

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1. Post-mortem changes in the concentrations of the following compounds in ox sternomandibularis muscles stored in nitrogen at 1°, 5° and 15° are reported: P_i creatine phosphate, hexose monophosphates, fructose diphosphate, triose phosphates, α -glycerophosphate, phosphoglycerates, lactate, ATP, ADP, AMP, NAD⁺ and total nucleotides. Some results obtained with muscles stored at 37° are included. 2. At the time the muscles were placed at controlled temperatures (about 1.5 hr. *post mortem*) the phosphorus in the compounds measured accounted for $91 \pm 6\%$ (S.D.) of the total acid-soluble phosphate. 3. The results indicated that at all temperatures the activities of the phosphorylase and phosphofructokinase steps limited the rate and the extent of post-mortem glycolysis. 4. The large variations in hexose monophosphate concentrations during storage indicated that the ratio of phosphorylase to phosphofructokinase activity varied considerably with time and temperature. 5. Between 3.5 and 7 hr. *post mortem* the rates of glycolysis and of ATP turnover were not slower at 5° than at 15°, and were probably faster at 1°. The significance of this finding is discussed.

In most previous studies on the post-mortem metabolism of mammalian muscle, relatively unspecific methods have been used to determine the concentrations of compounds containing phosphorus. For example, Bailey & Marsh (1952) used the amount of P_i released on treatment with cold alkali as a measure of triose phosphate, and the additional amount released by hot alkali as a measure of hexose diphosphate. Similarly, acid-labile phosphate from sources other than creatine phosphate (on occasions corrected for the amounts of triose phosphate and hexose diphosphate determined as above) has commonly been used as a measure of ATP concentration (e.g. Bate-Smith & Bendall, 1947; Bendall, 1951; Bailey & Marsh, 1952; Lawrie, 1953, 1960; Marsh, 1952, 1954).

Cassens & Newbold (1966) reported changes in pH and in the concentrations of P_i, creatine phosphate, acid-labile phosphate and alkali-labile phosphate in ox sternomandibularis muscles stored at 1°, 15° and 37° soon after slaughter. In later work (Cassens & Newbold, 1967a) they found that, whereas in the range 5–37° the pH of the muscles fell more slowly the lower the temperature, there was a

period of several hours during which the pH of muscle stored at 1° fell faster than that of muscle at 5°.

The present paper reports the results of a similar study in which, by specific methods of analysis, post-mortem changes in the concentrations of glycolytic intermediates and associated compounds, including the principal nucleotides, were followed during storage of ox muscle at 1°, 5°, 15° and, in a few instances, at 37°.

MATERIALS

Special chemicals. All nucleotides and substrates required for carrying out or checking the enzymic methods were obtained from the Sigma Chemical Co., St Louis 13, Mo., U.S.A.

Enzymes. The following enzymes, prepared in the laboratory, were of suitable quality for the enzymic analytical procedures; activities of the preparations are expressed as μ moles of substrate transformed/min. at 30°.

Glucose phosphate isomerase (EC 5.3.1.9) (about 200 units/mg.) was prepared from rabbit muscle by the method of Noltmann (1964).

Phosphoglucomutase (EC 2.7.5.1) (90 units/mg.) was prepared from pig muscle as described by Scopes (1964). An aqueous extract (pH 6.5) was treated first with acetone at –5°. The 42–65% (v/v) fraction was dialysed and brought

* Present address: Meat Research Institute, Low Temperature Research Station, Cambridge.

to 65% saturation with ammonium sulphate at pH 5.3; the precipitate was collected, dissolved in sodium acetate buffer, pH 5.3, and heated at 55° for 5 min. After the precipitate had been removed, ammonium sulphate was added to the solution until a slight turbidity developed. The enzyme crystallized on standing.

Glyceraldehyde phosphate dehydrogenase (EC 1.2.1.12) (80 units/mg.) was recrystallized several times from fraction V, prepared from either rabbit or pig skeletal muscle by the method of Czok & Bücher (1960).

Lactate dehydrogenase (EC 1.1.1.27) (220 units/mg. for lactate dehydrogenation at pH 10.0) was obtained from pig muscle, by a procedure similar to that described by Jécsai (1962) but including a heating step (pH 5.6, 52°, 10 min.), and was recrystallized three times. This enzyme was used only for the lactate determinations.

Creatine kinase (EC 2.7.3.2) (50 units/mg.) was prepared from rabbit muscle by the method of Noda, Kuby & Lardy (1955), in the presence of 5 mm- β -mercaptoethanol.

For preparing enzymes from yeast, 450 g. of vacuum-packed Dryco desiccated yeast (Compressed Yeast Co., Sydney, N.S.W., Australia) was stirred with 300 ml. of toluene and 1700 ml. of 0.1 M-tris base containing 1 mM-EDTA. The mixture was warmed to 35° and then stirred vigorously for 3 hr. while it cooled to room temperature. The paste was centrifuged at 20000g for 15 min. and the decanted aqueous layer, after being recentrifuged, was used for the preparation of alcohol dehydrogenase, hexokinase, or glucose 6-phosphate dehydrogenase.

Alcohol dehydrogenase (EC 1.1.1.1) (180 units/mg. at pH 7.5) was prepared by the method of Racker (1955) and crystallized five times.

Hexokinase (EC 2.7.1.1) (100–200 units/mg.) was prepared by the method of Darrow & Colowick (1962) to the stage of elution from bentonite.

Glucose 6-phosphate dehydrogenase (EC 1.1.1.49) (150 units/mg.) was prepared by a procedure involving an ammonium sulphate fractionation and an acid treatment as described by Ballard & Oliver (1964), an ethanol-magnesium acetate fractionation (Noltmann, Gubler & Kuby, 1961), an acetone-EDTA fractionation and chromatography on DEAE-Sephadex. Up to 6000 units were obtained from 450 g. of dried yeast.

All other enzymes (including ox-heart lactate dehydrogenase, which, because of its superior kinetic properties, was used in all systems involving reduction of pyruvate) were obtained from the Sigma Chemical Co.

METHODS

Ox sternomandibularis muscles obtained from the local abattoir soon after slaughter were transported without refrigeration to the laboratory, where they were freed from gross fat and connective tissue. About 1.5 hr. *post mortem* short lengths (6–10 cm.) of each muscle were placed in moist nitrogen at different temperatures and sampled at chosen intervals for pH determination and acid extraction. Measurements of pH were made as described by Newbold & Lee (1965).

Preparation of extracts. About 1 g. of muscle was homogenized for 30 sec. in about 15 ml. of cold 0.6 N-perchloric acid in a Marsh-Snow homogenizer (Marsh & Snow, 1950). After being filtered at 3°, the extract was at once neutralized to phenolphthalein with N-KOH, decanted from the resul-

tant potassium perchlorate precipitate and kept at 3° for several hours before a final decanting. About 2 ml. of the extract was kept aside for u.v. measurements, and to prevent bacterial action 1 drop of 20% (w/v) sodium azide was added to the remainder, which was then stored in the cold.

Chemical methods. Total acid-soluble phosphate, P_i , creatine phosphate, acid-labile phosphate from sources other than creatine phosphate, and alkali-labile phosphate were determined as described by Bailey & Marsh (1952).

Enzymic methods. All determinations were carried out in the presence of albumin (fraction V) at a concentration of 0.2 mg./ml.

Glucose 6-phosphate, glucose 1-phosphate and fructose 6-phosphate were measured sequentially on occasions by a combined procedure based on the methods described by Bergmeyer & Klotzsch (1963) and Hohorst (1963a), but usually the relevant enzymes were added together, the resulting NADPH formed being equivalent to the total hexose monophosphate. ATP and creatine phosphate were then determined as described by Lamprecht & Stein (1963).

ADP and AMP were determined essentially as described by Adam (1963). After the myokinase reaction had stopped, the content of fructose 1,6-diphosphate plus triose phosphates in the reaction mixture was determined as described by Bücher & Hohorst (1963). Since the concentration of fructose 1,6-diphosphate plus triose phosphates was near the limit of sensitivity of the method, no attempt was made to measure these compounds separately.

α -Glycerophosphate and lactate were determined by the methods described by Hohorst (1963b and 1963c respectively) except that, in both procedures, 50 mM-2-amino-2-methylpropane-1,3-diol buffer was used in place of the recommended α -glycine.

Pyruvate, phosphoenolpyruvate, 2-phosphoglycerate and 3-phosphoglycerate were determined with lactate dehydrogenase, pyruvate kinase, enolase and phosphoglyceromutase as described by Czok & Eckert (1963).

Creatine was measured at pH 8.5 essentially as described by Bernt, Bergmeyer & Möllering (1963). With 0.5 mg. of creatine kinase/ml., creatine phosphorylation was complete in 10 min.

NAD⁺ was measured by the method of Slater, Sawyer & Sträuli (1964).

Automation of methods. During the present experiments many of the above methods were adapted for use on the Technicon Autoanalyser, and the methods required little modification. P_i readings were taken 45 sec. after mixing with the reagents (as compared with 3 min. in the manual method), thus decreasing the amount of creatine phosphate hydrolysed in the acid reaction conditions.

Ultraviolet measurements. A modification of the Bendall & Davey (1957) differential spectrophotometric method for estimating total adenine and inosine (hypoxanthine) nucleotides was used. The data of these authors give $E_{259}^{1\text{mM}}$ about 14.1 for AMP. We measured the extinction of AMP and of ATP solutions in which the ATP, ADP and AMP concentrations had been determined enzymically, and obtained $E_{259}^{1\text{mM}}$ values of 15.4 and 15.2 respectively, which compare favourably with the generally accepted value of 15.4 (Bock, Ling, Morell & Lipton, 1956). When the spectra for both AMP and IMP were drawn and calibrated by using $E_{259}^{1\text{mM}}$ 15.4 for AMP and $E_{248.5}^{1\text{mM}}$ 12.2 for IMP (Beaven, Holiday & Johnson, 1955), the cross-over was found to occur at 250 m μ , where $E^{1\text{mM}}$ was 12.1 for each nucleotide. All u.v.-

absorbing substances were grouped as 'adenine nucleotide' and 'inosine nucleotide' and the following method was adopted. The extract was diluted in 10mM-potassium phosphate buffer, pH 7.5, and the extinction measured at 250m μ , 265m μ and 320m μ against an appropriate blank. A small amount of light-scattering was corrected for by subtracting 1.8 $\times$ E_{320} from the 250m μ reading and 1.6 $\times$ E_{320} from the 265m μ reading (corrections based on extrapolation from wavelengths above 310m μ) and from the corrected values the ratio E_{265}/E_{250} was calculated. The adenine nucleotide was then read off as a percentage of the total adenine nucleotide+inosine nucleotide from a linear graph. The ratio was 1.15 for 100% adenine nucleotide and 0.44 for 100% inosine nucleotide. The total nucleotide concentration was calculated from the corrected 250m μ reading.

Expression of results. To relate concentrations in the extract to concentrations in the muscle, the volume of extract equivalent to 1 g. of muscle was calculated from the total acid-soluble phosphate concentration in the extract, assuming that the total acid-soluble phosphate concentration in the muscle was 53.0 μ moles/g. (about 1.64 mg./g.) at all times (Cassens & Newbold, 1966).

RESULTS

Of the 15 muscles used in this study, four were stored at 1°, 15° and 37°, two at 1° and 15°, one at 1° and 5°, one at 5° and 15°, and seven at 1°, 5° and 15°. The pH of the muscle samples was determined at intervals during storage and measurements of the following were made on the extracts; P_i, creatine phosphate, hexose monophosphate (glucose 1-phosphate + glucose 6-phosphate + fructose 6-phosphate), fructose 1,6-diphosphate plus triose phosphates (as triose phosphate equivalents), lactate, ATP, ADP, AMP, NAD⁺, α -glycerophosphate, and

u.v. absorption. One or more of the above measurements were lacking for eight of the muscles; analyses were complete on the other seven. No AMP, lactate or u.v. measurements were made on any of the muscle samples stored at 37°.

'Initial values'

The means and standard deviations of the values at the time the muscle samples were placed at controlled temperatures (about 1.5 hr. *post mortem*) are given in Table 1. The mean initial pH (6.79) was appreciably below the mean values (close to 7.0) for the muscles used in each of the previous studies (Cassens & Newbold, 1966, 1967a) and the initial creatine phosphate concentration was correspondingly lower.

Changes up to 24 hr. post mortem

Figs. 1-4 and 6 show the means of all values obtained. Comparisons between 1°, 5° and 15° were basically the same whether all available results, or just the results for those muscles that were stored at all three temperatures, were used. The few results obtained with muscle held at 37° are also included in the Figures.

Changes in pH. Changes in pH are shown in Fig. 1. In the period 3.5-7 hr. *post mortem* the mean rate of pH fall at 1° was slightly faster than those at 5° or 15°. (Statistical tests gave *P* about 0.05 for the rate at 1° being different from that at 5° and also for the rate at 1° being different from that at 15°.) The rates at 5° and 15° were about the same whereas in the previous study of pH changes (Cassens & Newbold, 1967a) the rate at 5° was slower than that

Table 1. Concentrations of muscle constituents about 1.5 hr. post mortem

All results are given as means $\pm$ s.d. The pH of the 15 muscles at this time was 6.79 $\pm$ 0.12.

Constituent	No. of muscles analysed	Concn. (μ moles/g.)
Lactate	9	32.0 $\pm$ 9.0
P _i	15	19.6 $\pm$ 3.0
Creatine phosphate	15	3.7 $\pm$ 1.3
Hexose monophosphate	15	4.20 $\pm$ 1.0
Fructose 1,6-diphosphate + triose phosphates (as triose phosphate equivalents)	15	0.08 $\pm$ 0.05
ATP	15	4.90 $\pm$ 0.50
ADP	15	1.15 $\pm$ 0.22
AMP	11	0.26 $\pm$ 0.12
NAD ⁺	15	0.42 $\pm$ 0.06
Adenine nucleotides (u.v.)	9	7.10 $\pm$ 0.50
Inosine nucleotides (u.v.)	9	0.20 $\pm$ 0.10
α -Glycerophosphate	15	2.55 $\pm$ 0.9

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Fig. 1. Effect of temperature on the time-course of the pH change in ox muscle during the first 24 hr. *post mortem*. Points are mean values for 11, nine and 11 muscles at 1° (○), 5° (□) and 15° (△) respectively. Experimental details are given in the text.

Fig. 2. Relation between lactate concentration and pH in ox muscle during the first 24 hr. *post mortem*. Points are mean values for eight, nine and eight muscles at 1° (○), 5° (□) and 15° (△) respectively. Experimental details are given in the text.

at 15° and the rates at 1° and 15° were about the same. The rates at 1° were approximately the same in both sets of muscles.

Lactate. Lactate production, as in rabbit muscle (Bate-Smith & Bendall, 1956; Bendall, 1960), was linearly related to fall in pH (Fig. 2), and amounted to 60 μmoles/g./pH unit.

P_i, creatine phosphate and total hexose monophosphate. Changes in the concentrations of these constituents with time at different temperatures are shown in Fig. 3. There were no clear differences in the rates of loss of creatine phosphate at 1°, 5° and 15° (Fig. 3b).

Glucose 6-phosphate and fructose 6-phosphate made up all but a small fraction of the total hexose monophosphate at all times (up to 72 hr. *post mortem*) and temperatures. Previous deductions about post-mortem changes in the hexose 6-phosphate concentration (Cassens & Newbold, 1966), which were based on alkali-labile phosphate concentrations, are substantiated (Fig. 3c).

Concentrations of glucose 6-phosphate and of fructose 6-phosphate were measured individually in all extracts prepared from three muscles stored at 1°, 15° and 37° and in a few extracts from other muscles. Values for the ratio of glucose 6-phosphate to fructose 6-phosphate mostly fell in the range 3.3–4.3, the mean value (56 determinations) being 3.8. These values compare favourably with the equilibrium ratios of the phosphate esters obtained by Kahana, Lowry, Schulz, Passonneau & Crawford (1960) and by Özand & Narahara (1964). Both these groups of workers noted an effect of temperature on the equilibrium ratio; in the present study no definite effect of storage temperature or time on

Fig. 3. Effect of temperature on the time-course of changes in the concentrations of (a) P_i, (b) creatine phosphate and (c) hexose monophosphate (glucose 1-phosphate + glucose 6-phosphate + fructose 6-phosphate) in ox muscle during the first 24 hr. *post mortem*. Points are mean values for 14, nine, 14 and four muscles at 1° (○), 5° (□), 15° (△) and 37° (●) respectively. Experimental details are given in the text.

the ratio of glucose 6-phosphate to fructose 6-phosphate was apparent.

The relatively low concentrations of glucose 1-phosphate in the extracts did not permit accurate determination of the ratio of glucose 6-phosphate to glucose 1-phosphate. Values obtained varied between 10:1 and 25:1.

Fructose diphosphate and triose phosphates. The total concentration of fructose 1,6-diphosphate and triose phosphates was always very small, the mean value for all muscles at any temperature or time (up to 72 hr. *post mortem*) never exceeding 0.14 μmole/g., expressed as triose phosphate equivalents.

Adenine nucleotides. Changes in the concentrations of ATP, ADP, AMP and NAD⁺ with time are shown in Fig. 4. The specificity of the analytical

Fig. 4. Effect of temperature on the time-course of changes in the concentrations of (a) ATP, (b) ADP, (c) AMP and (d) NAD^+ in ox muscle during the first 24 hr. *post mortem*. Points are mean values for 14, nine, 14 and four muscles at 1° (○), 5° (□), 15° (△) and 37° (●) respectively, except in (c) where they are the means for ten, nine and ten muscles at 1° (○), 5° (□) and 15° (△) respectively. Experimental details are given in the text.

methods used was such that any ITP and IDP present (Bendall & Davey, 1957) would have been measured as ATP and ADP respectively.

Between about 3.5 and 8.5 hr. *post mortem*, the ATP concentrations at 1°, 5° and 15° fell linearly, the rate of decrease being on the average greatest at 1° and least at 15° (Fig. 4a), although in some muscles the rates at 1° and 15° were very similar. Subsequent changes were markedly different at 5° than at the other temperatures: in seven out of the nine muscles there was at 5° a pause in the ATP fall, though no pause occurred at 1° or 15°.

At all temperatures the ADP concentration remained about the same (Fig. 4b) until the ATP concentration had fallen considerably; the ADP

Fig. 5. Absorption spectrum (—), corrected for light-scattering, of a muscle extract in which inosine nucleotides and adenine nucleotides made up respectively 21% and 79% of the total adenine nucleotide concentration as determined from the 250 mμ and 265 mμ readings. Experimental details are given in the text. ○, Theoretical absorption spectrum of a nucleotide solution with an inosine nucleotide to adenine nucleotide ratio of 21:79, made to coincide with the absorption spectrum of the muscle extract at 250 mμ. △, Difference between the two absorption spectra.

then decreased slowly. Concentration of AMP was not measured in muscles kept at 37°, but in those kept at each of the other temperatures it increased appreciably (Fig. 3c), the greatest increase being at 1° and the least at 15°. At 1°, 5° and 15°, NAD^+ had decreased to about half its initial concentration by 24 hr. *post mortem*; a much faster loss occurred at 37° (Fig. 4d).

Ultraviolet measurements. In muscles kept at 1°, 5° or 15° (those kept at 37° were not examined in this respect), *post-mortem* decrease in the concentration of adenine nucleotide was accompanied by a corresponding increase in the concentration of inosine nucleotide, the total nucleotide concentration being essentially the same at all times and temperatures, and averaging 7.3 μmoles/g. It should be noted that inosine nucleotide as measured here includes any free inosine that may be present (Kerr & Seraidarian, 1945). The sum of the ATP, ADP, AMP and NAD^+ concentrations was always less than the concentration of adenine nucleotide estimated by u.v. measurement, the average difference between the two values being

0.6 $\mu\text{mole/g.}$, with no clear-cut dependence on time or temperature.

The u.v. spectrum of a typical muscle extract is shown in Fig. 5. This extract was neutralized at a pH-meter, and did not contain phenolphthalein; phenolphthalein interfered below 250 $\text{m}\mu$. The curve was corrected for light-scattering by a linear extrapolation from wavelengths above 310 $\text{m}\mu$, where the nucleotides do not absorb. The correction amounted to about 5% of E_{250} . From the ratio E_{265}/E_{250} it was calculated that in this extract adenine nucleotide accounted for 79% of the total nucleotide. The theoretical points (made to coincide at 250 $\text{m}\mu$ with the curve obtained for the extract) for a mixture in which adenine nucleotide makes up 79% and inosine nucleotide 21% of the total nucleotide are also shown. The difference curve suggests extra absorption by the extract in the region 270–290 $\text{m}\mu$, which may be due to free tryosine, tryptophan, peptides or proteins soluble in perchloric acid. Much of the extra absorption below 240 $\text{m}\mu$ can be ascribed to the carnosine present, as histidine absorbs in this range (Saidel, Goldfarb & Waldman, 1952).

α -Glycerophosphate. During the first 24 hr. *post mortem* the α -glycerophosphate concentration decreased at 5° and 15° but increased at 1° (Fig. 6). Similar results to those obtained here at 1° and 15° had previously been obtained for another set of ten muscles (M. R. Lloyd & R. P. Newbold, unpublished work). In all four of the muscle samples stored at 37° the α -glycerophosphate concentration increased by more than 1 $\mu\text{mole/g.}$ during the first 2.5 hr.

3-Phosphoglycerate, 2-phosphoglycerate, phosphoenolpyruvate and pyruvate. Concentrations of these compounds were measured in six muscles stored at 1°, 5° and 15°. The concentration of 3-phosphoglycerate was less than 0.05 $\mu\text{mole/g.}$ in the initial samples and increased after 16 hr. at 1° and 5° but not at 15°. Further increases after 24 hr. are shown in Table 2. Concentrations of 2-phosphoglycerate and of phosphoenolpyruvate were measured in ten extracts containing relatively large amounts of 3-phosphoglycerate. The average ratio of 3-phosphoglycerate to 2-phosphoglycerate was about 9, and of phosphoenolpyruvate to 2-phosphoglycerate about 3. Pyruvate concentrations were small (mostly less than 0.2 $\mu\text{mole/g.}$) at all times and temperatures and were not investigated systematically.

Acid-labile phosphate. Acid-labile phosphate was determined in most of the extracts prepared from the four muscles stored at 1°, 15° and 37° and the two muscles stored at 1° and 15°. The mean initial value of 12.1 $\mu\text{moles/g.}$ compared favourably with the value (12.8) obtained by Bodwell, Pearson & Spooner (1965), but we did not find the large discrepancy that these authors found between the

Fig. 6. Effect of temperature on the change in α -glycerophosphate concentration in ox muscle during the first 24 hr. *post mortem*. Points are mean values for 14, nine, 14 and four muscles at 1° (○), 5° (■), 15° (△) and 37° (●) respectively. Experimental details are given in the text.

estimates of ATP obtained from acid-labile phosphate measurements and the values obtained by an enzymic method. Their high ATP values determined by the bioluminescence method are not compatible with our u.v. measurements. Our acid-labile phosphate values were somewhat higher than the sum of $2 \times \text{ATP} + \text{ADP}$, the mean difference being 0.69 ± 0.07 (S.E.M.) $\mu\text{mole/g.}$ (89 determinations). No effect of time, temperature or ATP content on this difference was evident. Glucose 1-phosphate is acid-labile, and if assumed to be in equilibrium with glucose 6-phosphate it should amount to about 4% of the total hexose monophosphate; on this basis the mean contribution of glucose 1-phosphate to the acid-labile phosphate would have been about 0.15 $\mu\text{mole/g.}$

Alkali-labile phosphate. Cassens & Newbold (1966) based their estimates of hexose 6-phosphate on the assumption that the amount of alkali-labile phosphate (i.e. the amount of P_i liberated on hydrolysis in 0.2N-sodium hydroxide for 3 min. at 100°) was equivalent to 60% of the total amount of hexose 6-phosphate present (Kiessling, 1938; Robison & Macfarlane, 1941). In the present work alkali-labile phosphate was determined in 33 extracts (11 from each of three muscles stored 1°, 15° and 37°). Determined enzymically, the hexose 6-phosphate concentrations in these extracts were in the range 0.1–0.5 $\mu\text{mole/ml.}$ The mean ratio of the hexose 6-phosphate estimated from the amount of alkali-labile phosphate to the hexose 6-phosphate determined enzymically was 1.14 ± 0.04 (S.E.M.). There was no apparent effect of time or temperature of storage on this ratio.

Sum of phosphorus-containing fractions. It was noted by Cassens & Newbold (1966) that the total amount of phosphorus accounted for as P_i , creatine phosphate, $1\frac{1}{2} \times$ alkali-labile phosphate and acid-labile phosphate increased with time at 15° but not

Table 2. *Concentrations of muscle constituents 24, 48 and 72 hr. post mortem*

Unless otherwise noted, values are the means for the same six muscles.

Temperature	1°			5°			15°		
Time <i>post mortem</i> (hr.)	24	48	72	24	48	72	24	48	72
pH	5.89	5.71	5.71	5.87	5.74	5.74	5.64	5.64	5.62
Concn. (μ moles/g.)									
Lactate	84	92	91	87	95	94	98	99	97
P _i	27.6	31.6	33.7	28.6	31.5	33.9	36.9	39.6	41.0
Creatine phosphate	0	0	0	0	0	0	0	0	0
Hexose monophosphate	8.4	5.4	5.3	9.3	7.8	6.9	4.7	4.5	4.1
Fructose 1,6-diphosphate + triose phosphates (as triose phosphate equivalents)	0.07	0.05	0.04	0.06	0.04	0.03	0.04	0.03	0.03
ATP	0.34	0.20	0.17	0.52	0.18	0.14	0.25	0.19	0.19
ADP	0.49	0.36	0.31	0.53	0.33	0.32	0.37	0.33	0.28
AMP	0.32	0.22	0.20	0.42	0.22	0.20	0.23	0.23	0.17
NAD ⁺	0.21	0.16	0.13	0.25	0.17	0.14	0.24	0.10	0.07
Adenine nucleotides (u.v.)*	2.05	1.85	1.65	2.50	2.05	1.65	1.90	2.20	2.05
Inosine nucleotides (u.v.)*	5.15	5.50	5.45	4.80	5.20	5.20	5.00	4.80	4.60
α -Glycerophosphate	2.55	2.30	1.55	1.80	1.50	1.05	1.20	0.75	0.60
3-Phosphoglycerate	0.53	0.88	0.78	0.17	0.27	0.13*	0.01	0.06*	0.01*

* Means for four muscles only.

at 1°. In the present work similar trends to the above were apparent in the total amount of phosphorus accounted for as P_i, creatine phosphate, glucose 6-phosphate, fructose 6-phosphate, glucose 1-phosphate, 2 × ATP and ADP. Part of the difference in these sums at 1° and 15° is due to α -glycerophosphate, which is acid- and alkali-stable and increases at 1° but decreases at 15° (Fig. 6). At 5°, as at 15°, the sum increased with time. The mean values of this sum were 2–3 μ g.atoms/g. lower than of the sum based on the phosphate measurements made in the previous study. This is partly explained by the overestimates of hexose 6-phosphate and of 2 × ATP + ADP + glucose 1-phosphate by using alkali- and acid-labile phosphate values respectively.

At 1.5 hr. *post mortem* the total amount of phosphorus in P_i, creatine phosphate, hexose monophosphate, fructose 1,6-diphosphate, triose phosphates, ATP, ADP, AMP, NAD⁺, α -glycerophosphate and inosine nucleotide (assumed to be all IMP), namely the 'identified phosphorus', accounted for 91 ± 6% (s.d. for nine muscles) of the total acid-soluble phosphate. The additional amount of phosphorus in the phosphoglycerates and phosphoenolpyruvate was initially very small (less than 0.2% of the total acid-soluble phosphate). No attempts were made to identify other phosphorylated compounds; part of the unidentified phosphorus may be attributable to the compound isolated by Satchell & Freeman (1964).

There were no significant changes in identified phosphorus at later times *post mortem*.

Creatine. Measurements of free creatine were made on the extracts of two muscles. There was no change in the sum of free creatine + creatine phosphate during storage for up to 72 hr. *post mortem* at any temperature. The mean values for the total creatine in these two muscles were 30.0 ± 0.6 (s.d.) μ moles/g. (35 determinations) and 29.4 ± 0.6 (s.d.) μ moles/g. (36 determinations) respectively. Some later muscles, not included in the present set of 15, have had total creatine concentrations considerably higher than this, up to 39 μ moles/g.

Changes between 24 and 72 hr. post mortem

Mean values for various constituents 24, 48 and 72 hr. *post mortem* are given in Table 2. It can be seen that accumulation of 3-phosphoglycerate occurred at the lower temperatures. The ultimate pH in these six muscles was reached at 15° by 24 hr. *post mortem* and at 1° and 5° by 48 hr. *post mortem*. After the ultimate pH was reached there was little further change in the ATP, ADP or AMP concentration; on the other hand the α -glycerophosphate concentration decreased at all temperatures, and there was further loss of NAD⁺.

Considerable breakdown of IMP to hypoxanthine can be expected by 72 hr. at 15° (Lee & Webster, 1963). Hypoxanthine has a markedly different u.v. spectrum from inosine or IMP, and thus the u.v. measurements would be upset. This can explain the apparent higher adenine nucleotide and lower total nucleotide values at 15° after 24 hr.

DISCUSSION

Of the 12 enzyme reactions involved in the conversion of glycogen into lactate, two, the phosphorylase and phosphofructokinase reactions, have been closely implicated in the regulation of the rate of glycolysis in living muscle (see Helmreich & Cori, 1965). The present results indicate that these same two reactions are of prime importance in the regulation of glycogen breakdown *post mortem*.

In the following discussion, phosphorylase activity and phosphofructokinase activity refer to the net 'throughput' at each of these steps, i.e. to the activities apparent from consideration of changes in lactate and hexose monophosphate concentrations (see below). Allowance for any additional activity, which would have been balanced with phosphorylase by re-formation of glycogen by the UDP-glucose-enzyme system, and with phosphofructokinase by re-formation of fructose 6-phosphate by fructose 1,6-diphosphatase (Krebs & Woodford, 1965), was not possible.

It is apparent that both the rate and the total amount of lactate production *post mortem* were determined almost completely by activity at the phosphofructokinase step, for (1) changes in concentration of any of the intermediates between fructose 6-phosphate and lactate, including α -glycerophosphate, were small compared with concomitant increases in lactate concentration, and (2) substantial amounts of fructose 6-phosphate and ATP were present during the period of pH fall, and even after glycolysis had ceased the ATP concentration was considerably greater than the K_m of the enzyme for ATP (as measured at pH 8). The complex interaction of substrates and of AMP on the activity of phosphofructokinase at pH values below 7.5 has been little investigated (see Trivedi & Danforth, 1966), but it is clear that the enzyme may exhibit only a minute fraction of its potential activity when the pH is below 7. Bate-Smith & Bendall (1949) suggested that loss of phosphofructokinase activity might be responsible for the cessation of glycolysis. The present results support this view, at least for muscles that reach an ultimate pH of about 5.65 (present range 5.61–5.68).

The enzymic methods used did not distinguish between ADP and IDP or between ATP and ITP, but the u.v. results indicate that most of the enzymically determined di- and tri-phosphates were adenine nucleotides. The persistence of small amounts of nucleoside di- and tri-phosphates and of AMP in the muscle after glycolysis had ceased suggests that these compounds were not available to various enzymes; for instance, that AMP was not available to AMP deaminase, nor the triphosphate(s) to adenosine triphosphatases. Thus it seems possible that the primary reason for cessation

of lactate production could be the unavailability of the triphosphates to phosphofructokinase (ITP can replace ATP in the reaction).

Activity at the phosphorylase step was also limited, for the ratio of the hexose monophosphate concentration to the P_i concentration never approached the theoretical equilibrium value even after the ultimate pH was reached, whereas the substrate/product ratios for the phosphoglucosmutase and glucose phosphate isomerase reactions, when measured, were always close to the theoretical equilibrium values. Also, since the hexose monophosphate concentration did not rise after the ultimate pH was reached, it can be concluded that there was no further phosphorylase activity after phosphofructokinase activity had stopped. In view of the results obtained with other ox sternomandibularis muscles (Newbold & Lee, 1965) it seems unlikely that complete exhaustion of the glycogen reserves was responsible for this cessation of phosphorylase activity.

The adenosine triphosphatases, like phosphofructokinase and phosphorylase, also exhibited only a small fraction of their total potential activity. As has been indicated above, the limited activities of all these enzymes are not attributable to lack of substrates in the muscle, and thus some other factors must be involved. These may include the interconversion of active and inactive forms of the enzyme(s), the presence of various activators and inhibitors in the environment, and the restricted access of the substrates to the enzymes (Helmreich & Cori, 1965).

Since the fall in pH is linearly related to lactate production (Fig. 2), phosphofructokinase activity is reflected in the pH curves (Fig. 1), and can be assessed from changes in either lactate or pH. Similarly, phosphorylase activity can be calculated by also taking into account changes in hexose monophosphate concentration, and total adenosine triphosphatase activity by considering the glycolytic resynthesis of ATP and the changes in total 'high-energy' phosphate concentrations ($2 \times \text{ATP} + \text{ADP} + \text{creatine phosphate}$). The activities of these enzymes would be expected to fall with lowering temperature. However, in the period 3.5–7 hr. *post mortem*, their activities were not lower at 5° than at 15°, and were probably somewhat greater at 1°. Thus it is apparent that the decrease in metabolic rate expected as a result of lowering the temperature was counteracted by some factor(s) that accelerated the metabolism. The phosphorylase step was accelerated the most, as is evident from the increase in hexose monophosphate concentrations at the lower temperatures. It is noteworthy that during this period the AMP concentration rose only slightly at 15°, but more at 5°, and even more at 1°. AMP is known to activate phosphorylase *b* and to

stimulate phosphofructokinase *in vitro*; this increase in AMP may therefore be the factor responsible for the acceleration of the phosphorylase and phosphofructokinase steps at the low temperatures. In this event, the acceleration of glycolysis at low temperatures could be a result of a stimulation of total adenosine triphosphatase activity.

In addition to its effect on metabolic rate, temperature is known to have a marked effect on the time-course and the amount of post-mortem shortening in ox sternomandibularis muscle. When placed at temperatures below about 14° soon after slaughter, this muscle starts to shorten immediately and continues to shorten for several hours (Lockyer & Hagyard, 1963). Assuming that shortening always involves myofibrillar adenosine triphosphatase activity, it can be concluded that myofibrillar adenosine triphosphatase was activated at the lower temperatures. This would explain why the total adenosine triphosphatase activity was not less at these temperatures than at 15°.

Both the rate and the total amount of shortening, like the stimulation of total adenosine triphosphatase activity and of glycolysis, are greater at 1° than at 5° (Cassens & Newbold, 1967b). Compared with the effects observed at low temperatures, even more rapid rates of shortening and greater increases in ATP turnover and glycolytic rate are observed when muscle frozen pre-rigor is thawed (Bendall, 1960) and when muscle is stimulated electrically (Cain & Davies, 1962; Cori, 1956; Karpatkin, Helmreich & Cori, 1964). Hence an acceleration of glycolysis above a basal rate in either resting or post-mortem muscle appears to be closely related to activation of myofibrillar adenosine triphosphatase.

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