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RIGOR MORTIS AND ADENOSINETRIPHOSPHATE

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Erdös (1943), working in Szent-Györgyi's laboratory, has shown that the destruction of adenosinetriphosphate (ATP) and the hardening of rabbit's muscle follow parallel courses during the development of rigor mortis. Taking into account the known importance of ATP in muscular contraction, the fact that myosin itself can apparently act as an adenosine triphosphatase (Engelhardt & Ljubimova, 1939; Bailey, 1942), and the effect of ATP in vitro on myosin (or actomyosin) sols and artificial fibres (Dainty, Kleinzeller, Lawrence, Miall, Needham, Needham & Shen, 1944; Szent-Györgyi, 1945), Erdös and Szent-Györgyi conclude that lack of ATP is in fact responsible for the stiffening of the muscle. Although lactic acid formation generally accompanies the development of rigor in muscle, Claude Bernard (1877) and later Hoet & Marks (1926) demonstrated that rigor could occur in the complete absence of acid production. Hoet & Marks therefore suggested that the factor providing common ground between the acid and alkaline types of rigor was the disappearance of hexose phosphate from the muscle. Smith (1930) pointed out that, while the facts clearly required the assumption of a third change to which both stiffening and lactic acid were related, many further possibilities of chemical change existed.

The present work confirms that breakdown of ATP is correlated with the onset and development of rigor in normal animals. It extends Erdös's observations to animals with depleted reserves of glycogen, and shows that in these cases also there is a relation between stiffening and disappearance of ATP. The same relation does not hold between stiffening and lactic acid production even when the acid production is of normal magnitude, but the coincidence observed by Bate-Smith (1939), between the initiation of rigor and the attainment of a pH in the neighbourhood of 6·2, is confirmed. A mechanism is suggested for this coincidence through the interaction of ATP breakdown and the glycolytic cycle in muscle. It would appear that the breakdown of ATP is, in fact, the 'third change' which in 1930 remained to be identified.

METHODS

Preparation and extraction of muscle. The rabbits used in these experiments were killed by a blow on the nape of the neck and bled thoroughly after decapitation. The psoas major muscles were then removed as rapidly as possible, one being used for the determination of adenyl-polyphosphate phosphorus (i.e. the labile phosphorus of ATP and ADP) and the other for measurement of the modulus of elasticity by the method of Bate-Smith (1939). It has been shown by Bate-Smith (1939) that the development of rigor takes place at an equal rate in the left and right psoas muscles, both in 'normal' rigor and in 'alkaline' rigor following insulin treatment.

For the analysis of the phosphorus compounds, samples weighing 1–2 g. were cut progressively from the proximal end of the muscle at the times shown in the tables, the first sample being cut within 7 min. of death. The samples were then plunged into 5 c.c. ice-cold 10% trichloroacetic acid (TCA) in a mortar, and ground to a fine pulp with sand. The pulp was washed on a filter with 4×5 c.c. 5% TCA, and the filtrate collected in a 50 c.c. volumetric flask. The pH was then adjusted to 8·0 by the addition of N-NaOH, and the volume made up to 50 c.c.

Estimation of phosphorus content. The 'inorganic-P' content was estimated on 2 c.c. aliquots of each extract by the colorimetric method of Allen (1940), the final volume of the coloured solution being 25 c.c. in each case. The colour intensity was measured after 25 min. on a Spekker photometer, using a red filter. In this way all the photocreatine present is hydrolysed to inorganic phosphate and creatine, and the so-called 'inorganic-P' therefore includes phosphocreatine-P.

The '7 min.-P' was also estimated on 2 c.c. aliquots, after hydrolysis in N-HCl for 7 min. at 100° C.

The 'total P' was estimated on 1 c.c. aliquots after hydrolysis with perchloric acid by the method of Allen (1940).

The adenyl-polyphosphate phosphorus content is given approximately by difference between the '7-min.-P' and 'inorganic P'. The former, however, may contain a small amount of phosphate arising from the partial breakdown of other phosphate esters, most of which require more than 2 hr. for complete hydrolysis under these conditions. During the first 14 min. the hydrolysis curve of these compounds is virtually a straight line, and hence, by carrying out a 14 min. hydrolysis in N-HCl, an estimate can be obtained of the non-polyphosphate phosphorus appearing in the first 7 min. The polyphosphate values in the tables and figures have been corrected to take account of this.

Estimation of lactic acid content and pH *values.* The lactic acid content was estimated on 30 c.c. aliquots of the extracts by the method of Friedemann & Graeser (1933), after treatment with copper-lime mixture to remove reducing compounds.

The pH of the samples was estimated by means of the glass-electrode on 1–2 g. of minced muscle, moistened with M/200-sodium iodoacetate. Owing to the small amount of muscle available it was not possible to measure the pH values side by side with the phosphorus values, and the stated pH values are therefore calculated from the final pH, the lactic acid values and the buffering capacity. The latter was measured by titration with 0·05 N-HCl or -NaOH, of the same samples as those used for determination of pH. The pH values so calculated are correct but for an amount due to escape of CO_2 which makes them too high by $> 0{\cdot}2$ pH unit in the early post-mortem period. Thus the pH value calculated for muscle completely free from lactic acid is always about 7·6, whereas the value is more probably 7·4 (cf. Bate-Smith, 1938).

Variations in composition of samples taken simultaneously and errors of determinations. In order to estimate the magnitude of errors due to these factors a rabbit was killed and one psoas muscle removed immediately. This was then cut into six portions, weighing 1–2 g. each of which was plunged immediately into 10% trichloroacetic acid. The samples were immediately extracted and the phosphorus content estimated. The mean error of estimation of the inorganic, total '7-min.' and total P values amounted to less than 2% of the values in each case. On the other hand, the error in the polyphosphate-P values, obtained by difference, was found to be at least $\pm 5\%$. becoming greater as the values become smaller, thus effectively limiting the reliability of the method to estimation of values not less than 0·05 mg. polyphosphate-P/g.

RESULTS

The results of four experiments in which the rates of onset and development of rigor were very different are given in Table 1. The phosphate values are expressed as mg./g. of fresh muscle. The unidentified phosphorus content (UP), which is the difference between the polyphosphate + inorganic phosphorus and the total, is also given in the table. This fraction contains mainly the phosphorus of adenylic acid, of hexose-1-, -6- and -di-phosphates and triose-phosphate. The lactic acid values are expressed as mg. lactic acid per g. of muscle.

In Figs. 1 and 2 the polyphosphate-P values, the pH and the modulus (*E*) values are plotted against time. The polyphosphate-P remaining at any time is plotted as mg. P/g. of muscle, and the modulus as a percentage of the final modulus attained.

TABLE 1. Changes in the phosphorus compounds during development of rigor mortis in rabbit muscle

	Phosphorus and lactic acid values						Modulus	
	Time after death (min.)	Inorganic P (mg./g.)	Polyphosphate P) (mg./g.)	Unidentified P (mg./g.)	Total P (mg./g.)	Lactic acid (mg./g.)	pH (calc.)	Time after death (min.)	*E* g./cm.²
Exp. 5. 3 kg. female. Normal. Well fed. Intraperitoneal injections of glucose	4	0·84	0·33	0·60	1·77	3·25	6·91	22	610
	43	0·85	0·37	0·56	1·78	2·85	6·99	128	715
	113	0·91	0·32	0·58	1·81	3·85	6·78	240	810
	236	0·82	0·26	0·57	1·65	4·90	6·55	375	4600
	368	0·95	0·11	0·61	1·67	6·90	6·08	540	4600
	556	1·11	0·08	0·56	1·75	8·15	5·74	600	4600
	1440	1·33	0·00	0·41	1·74	8·75	5·58	1440	5000
Exp. 6. 2·5 kg. female. Starved 40 hr. Injected with insulin at 8.15 a.m. and at intervals thereafter. Died at 5.40 p.m. Total dose of insulin = 121 units	4	1·24	0·36	0·19	1·79	0·35	—	15	2440
	16	1·33	0·24	0·22	1·79	0·165	—	44	6600
	33	1·45	0·15	0·18	1·78	0·10	—	83	8000
	78	1·59	0·17	0·14	1·90	0·10	7·26	1440	8000
	963	1·63	0·07	0·09	1·79	0·55	7·25		
Exp. 8. 2 kg. female. Normal. Fed. Intraperitoneal injections of glucose	5	1·16	0·34	0·50	2·00	3·80	6·86	23	900
	55	1·46	0·15	0·41	2·02	5·97	6·45	37	1800
	61	1·55	0·16	0·41	2·12	5·45	6·54	56	3000
	82	1·61	0·11	0·39	2·11	6·09	6·43	73	3650
	130	1·58	0·11	0·39	2·08	6·75	6·28	91	4050
	1440	1·91	0·01	0·19	2·11	8·20	5·91	110	4250
	330	—	—	—	—	—	6·22	360	4700
								1440	4900
Exp. 9. 2·5 kg. male. Normal. Well fed	4	0·71	0·47	0·69	1·89	3·96	6·83	25	890
	229	0·93	0·36	0·71	2·00	6·25	6·43	93	1000
	304	0·85	0·23	0·94	2·02	6·65	6·35	260	1100
	329	0·93	0·17	0·90	2·00	7·44	6·20	337	1950
	344	0·90	0·17	0·91	1·98	7·70	6·14	347	3100
	371	0·86	0·21	0·86	1·93	8·05	6·06	364	4450
	444	0·96	0·14	0·92	2·02	8·75	5·90	382	6100
								398	7000
								1440	7400

DISCUSSION

The outstanding feature of all the experiments, not only of the four typical examples quoted but also of others which for the sake of brevity have not been detailed, is the close correspondence between the rate of disappearance of the adenyl-polyphosphate-P fraction and the rate of increase of the modulus of elasticity. In the three experiments in which lactic acid production occurred,

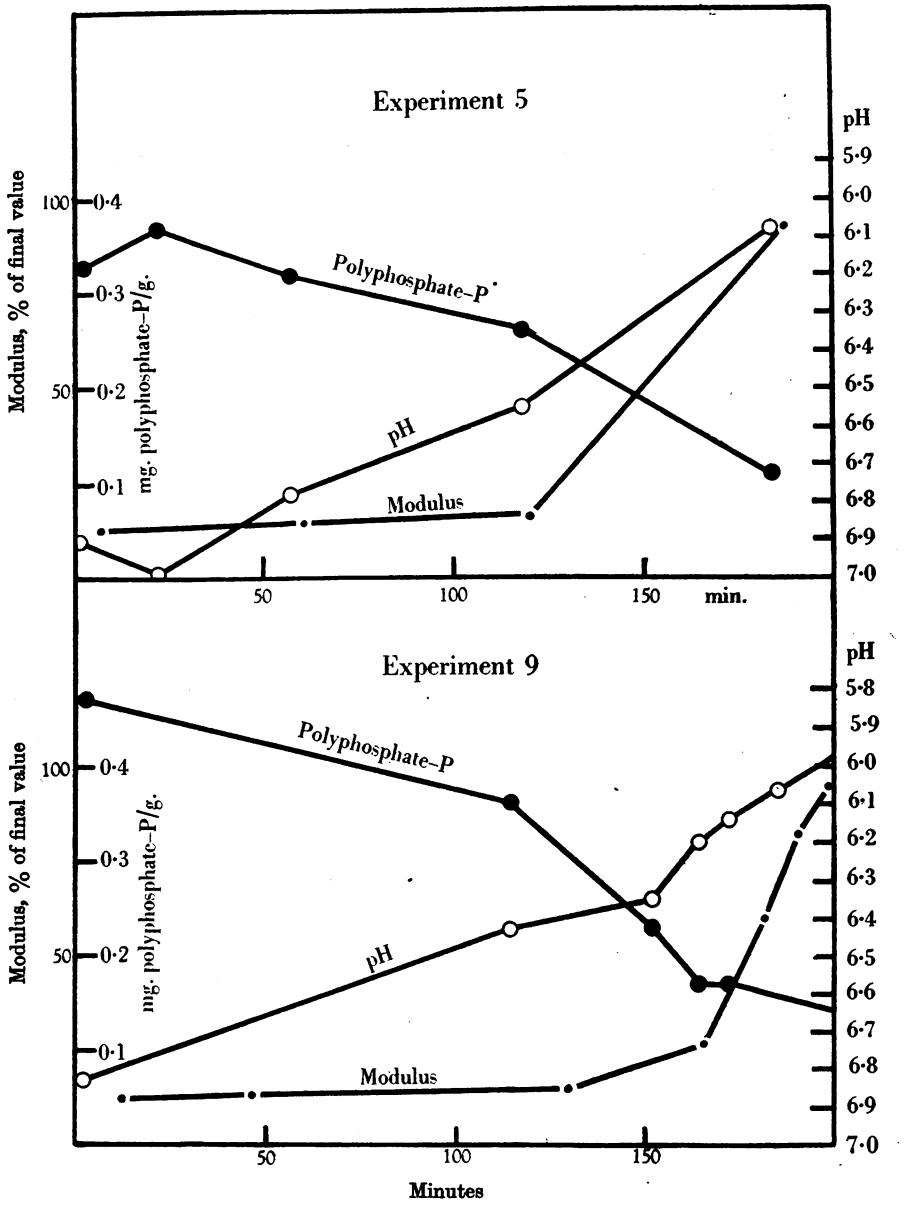

Fig. 1. The modulus, polyphosphate-P and pH values of rabbit psoas muscle during development of rigor mortis. Exp. 5, normal rabbit; ultimate pH 5·58. Exp. 9, normal rabbit; ultimate pH 5·60.

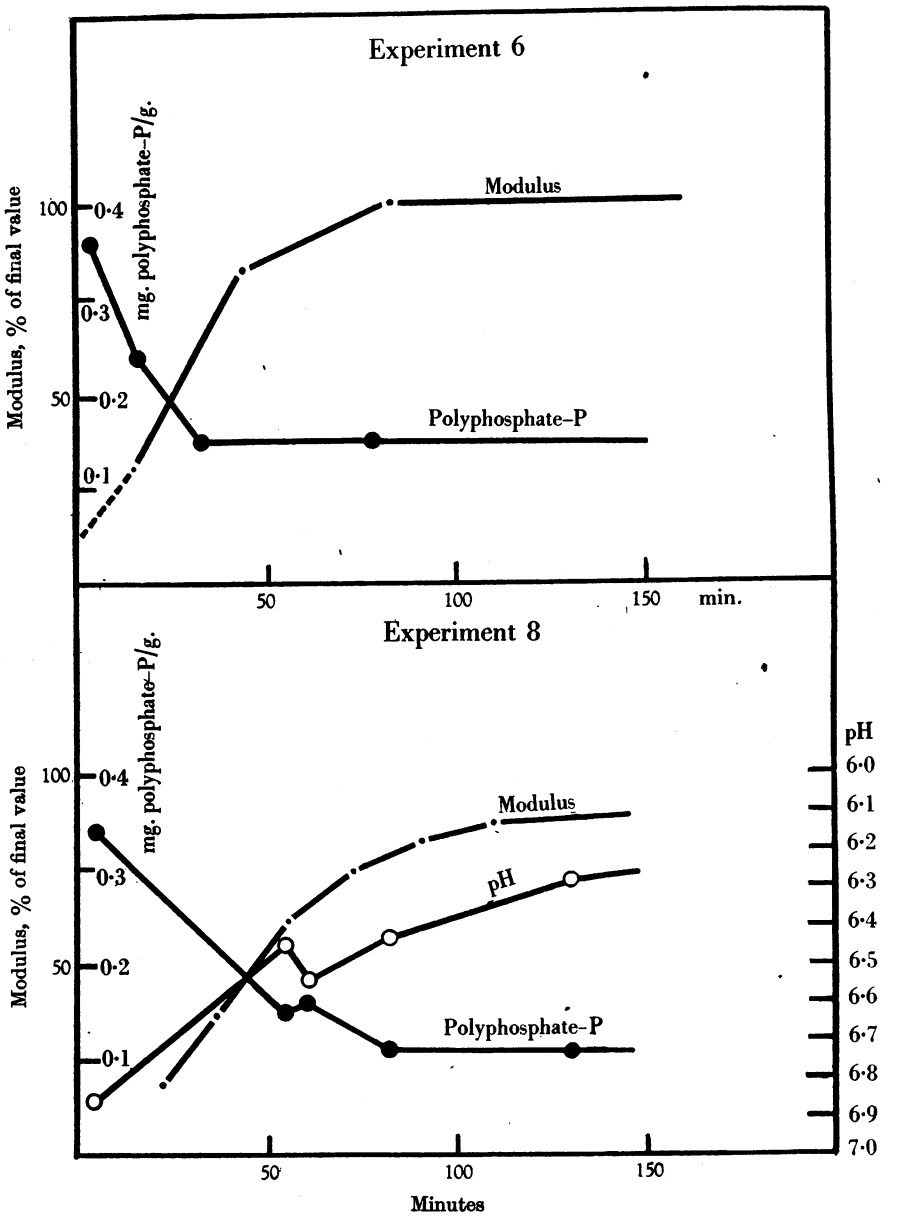

Fig. 2. The modulus, polyphosphate-P and pH values of rabbit psoas muscle during development of rigor mortis. Exp. 6, insulinized rabbit; ultimate pH 7·25. Exp. 8, normal rabbit; ultimate pH 5·91.

and in which the rapid stage of rigor was delayed, an increase in rate of breakdown of polyphosphate coincided with the rapid onset of rigor. In the fourth experiment (Exp. 6), in which there was no production of lactic acid, no delay occurred in either the onset of the rapid stage of rigor or the rapid breakdown of polyphosphate.

The production of lactic acid when it does occur, is continuous from the moment the first sample is taken and does not as a rule cease when rigor is completed. There is, however, some degree of acceleration during the rapid stage of rigor which is probably secondary to the rapid breakdown of polyphosphate at this stage. There is every reason to suppose, therefore, that the process of stiffening is directly connected with the decrease in the polyphosphate fraction, and is in fact, as Erdös (1943) deduced, attributable to the disappearance of ATP from the system.

The plan of our experiments did not permit of determinations of ATP; the behaviour of ATP and of ADP must therefore be assumed from the evidence available.

The following enzymes have been claimed to be present in muscle: an ATP-ase, associated with myosin (Engelhardt & Ljubimova, 1939) and active in the breakdown of ATP to ADP at pH 7 and more alkaline reactions, and myokinase, which catalyses the breakdown of ADP to adenylic acid, possibly with some resynthesis of ATP (Kalckar, 1943). Sakov (1941) has further isolated a transesterifying enzyme, with maximum activity at pH 7, which catalyses the transference of phosphate from ATP to fructose-6-phosphate (Neuberg ester) and a mineralizing enzyme which catalyses the production of inorganic phosphate from ATP and ADP at a pH optimum of 6, the reaction proceeding feebly at pH 7. In experiments with dialysed muscle juice to which ATP and fructose-6-phosphate were added, Sakov found that at pH 7 the main reaction was transesterification while at pH 6·0 inorganic phosphate production predominated.

Besides these phosphorylases and phosphatases, it may be assumed that under the conditions of our experiments the anaerobic glycolytic cycle will be operative. In this cycle, the breakdown of glycogen to lactic acid (2 mol.) is coupled with resynthesis of 3–4 mol. of ATP from ADP or adenylic acid (Lipmann, 1941; Needham, 1942).

From this it is evident that the conditions may be very different, depending on whether glycolysis does or does not occur. When the glycolytic cycle is unable to operate owing to total absence of glycogen, as in Exp. 6, the breakdown of polyphosphate, unaccompanied by any resynthesis, is rapid from the moment of death, but slows down markedly when about 50% of the labile P has disappeared. Even after 24 hr. 25% of the original polyphosphate-P was still present in the muscle. Further experiments are required to determine the precise nature of this polyphosphate-P.

On the other hand, when glycolysis is fully operating, as in Exps. 5, 8 and 9,

the first stages of the polyphosphate curve will represent a balance between breakdown and synthesis. Even at this stage, however, the conditions favour breakdown, which proceeds slowly for about 4 hr. in Exps. 5 and 9, but rapidly from the outset in Exp. 8. In Exps. 5 and 9 the rate of breakdown increases fivefold after this initial lag period. This increase begins when the pH is about 6·5. It is during the period of breakdown of polyphosphate, not during the preceding lag period, that the significant change in modulus occurs. If resynthesis of ATP is proceeding this change in rate of net breakdown might be caused by a decrease in rate of resynthesis or by an increase in rate of total breakdown. However, the rate of production of lactic acid, and hence of resynthesis, does not decrease, but rather increases, during this period. The net increase in breakdown must, therefore, be due to the increase in gross breakdown. It is permissible to attribute this to the activity of the mineralizing enzyme described by Sakov, which becomes greater as pH 6·0 is approached. If at this stage the glycogen in the muscle is exhausted, so that the pH remains in the neighbourhood of 6·0, as in Exp. 8, the breakdown of polyphosphate appears to proceed rapidly to completion, but if the pH falls further (Exps. 5 and 9), the reaction slows down and appreciable amounts of polyphosphate may still be present after 24 hr.

Thus, although it is clear that destruction of glycogen, leading to production of lactic acid and fall in pH, is not directly responsible for the increase in modulus, the moment at which the modulus begins to increase and its rate of increase are both clearly dependent upon the pH of the system and the prevailing rate of glycolysis, because these are also the factors on which the rate of destruction of ATP depends. As we have seen, it is this destruction of ATP and this alone with which the onset of rigor can be associated, whether or not accompanied by glycolysis.

The results substantiate Erdös's conclusions. As we have seen, however, the circumstances of rigor are more complicated than would be supposed from the linear relations which he depicts both for the breakdown of ATP and for the onset of stiffening with time after death.

Factors determining the time-course of rigor. There is considerable variation in the time after death at which rigor mortis sets in. We have observed that in animals which are relatively passive before stunning, the delay before the onset of rigor is longer than in animals which struggle violently, and this delay becomes still longer in narcotized animals (Bate-Smith, 1939). It seems likely that two factors may be responsible for the observed variations: the actual production of lactic acid in the muscles as a result of struggling; and a subsequent and sustained increase in the rate of glycolysis. Any factor which has an effect on the metabolic rate of resting muscle can, in fact, be assumed to influence the rate of production of lactic acid immediately post mortem, and thereby affect the period of delay before rigor sets in.

Shortening during rigor. Shortening occurs only when rigor develops at a pH higher than 6·2 and is not a normal concomitant of rigor. It is probable that below this pH the muscle is no longer capable of shortening when stimulated, so that the stimulus (whatever it may be) that causes contraction when rigor sets in above pH 6·5 may always be present at the moment rigor sets in whether a response follows or not. In isolated muscles it is observed that, when rigor is precipitate the shortening is greater and accompanied by greater development of tension. An extreme instance of this is shown in Fig. 3, which is a reproduction of the tracing of the psoas muscle in Exp. 8, illustrating the behaviour on application and removal of a load of 50 g./cm.^{2} immediately after death. The muscle was then at pH 6·7, and rapidly going into rigor. A total shortening of 16% occurred. The tension developed by this shortening, calculated from the magnitude of the response to the applied load, is of the order of 250 g./cm.^{2},

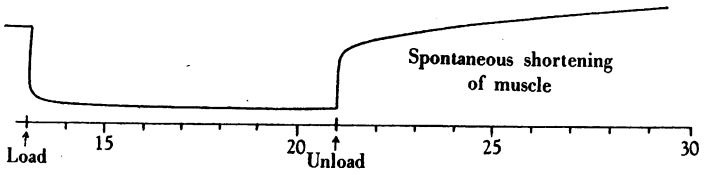

Fig. 3. Form of extension and recovery of rabbit psoas muscle resulting from application and removal of load, showing both the immediate and delayed phases of deformation and the effect of shortening on recovery curve as rigor is initiated. (Redrawn from kymograph tracing of Exp. 8.)

i.e. about 1/20th only of the maximum force that the muscles are capable of developing (cf. Haxton, 1944). It is to be remarked again that this represents an unusual degree of shortening, corresponding to the unusually high pH at which rigor sets in.

Only rarely in practice, at least at the slaughter house, does rigor take a course other than that of Exps. 5 and 9, since the glycogen content is usually sufficient to lower the pH to 5·6 or below. The rabbits available for the present work, in contrast to those used before the war, tended to have an abnormally high ultimate pH, due no doubt to the low level of feeding which it had been necessary to maintain during their rearing. After a fortnight's feeding with a supplement of starch the glycogen content improved to the extent indicated in Exps. 5 and 9.

SUMMARY

1. The stiffening of mammalian muscle during the onset of rigor mortis is correlated with a decrease in adenyl polyphosphates which is specifically interpreted as a decrease in adenosine triphosphate. Thus the earlier observation of Erdös (1943) is confirmed.

2. The correlation is observed both in normal ('acid') and in 'alkaline' rigor. In the former, acid production also runs parallel. An explanation of this

secondary correlation, and also of the normal lag period before onset of rigor, is put forward in terms of the variation with pH of the activity of the polyphosphatases known to be present in muscle.

3. Shortening occurs only if the pH of the muscle is greater than about 6·2 when stiffening sets in. This pH is unusual in animals with a normal reserve of glycogen. The force associated with shortening, when it occurs, is small in comparison with the absolute force developed during voluntary contraction.

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