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THE BUFFERING OF MUSCLE IN RIGOR; PROTEIN, PHOSPHATE AND CARNOSINE

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In the course of rigor mortis the *p*H of normal mammalian muscle falls from the value in living muscle of 7·0–7·5[1] to a value between 5·5 and 6·0. The extent of the fall is determined by two factors:

(*a*) the algebraic sum of acidic and basic equivalents formed post-mortem; and

(*b*) the buffer curve of muscular tissue.

While it is well known that the breakdown of glycogen to lactic acid is mainly responsible for the change in *p*H, comparatively little is known about the buffering of muscle, the substances responsible for it, and the variations normally met with in the concentrations of these substances.

The buffering in full rigor will differ from that of living muscle in two important respects. First, practically all esterified phosphate will be hydrolysed to orthophosphate; and secondly, any bicarbonate present at death will be destroyed. The buffering system is, therefore, much simpler than that of living muscle and more susceptible of analysis, whilst still affording much information relevant to the conditions present *in vivo*.

It is known that the muscle proteins, myosin in particular [Weber, 1934], are effective buffers in this range, but, as Meyerhof & Lohmann [1926] point out, only about one-half the buffering can be accounted for by the proteins in frog's muscle, and in mammalian muscle in rigor this

[1] The actual value is a matter of dispute. In inert muscle it is probably as high as 7·55 [Voegtlin *et al.* 1934], but Fenn & Maurer [1935] calculate that the *internal* *p*H may be only 6·9.

is reduced to as little as one-third, as can be seen in Fig. 1 which shows the titration curves of a specimen of minced ox muscle and of an ultrafiltrate of the juice expressed from it.

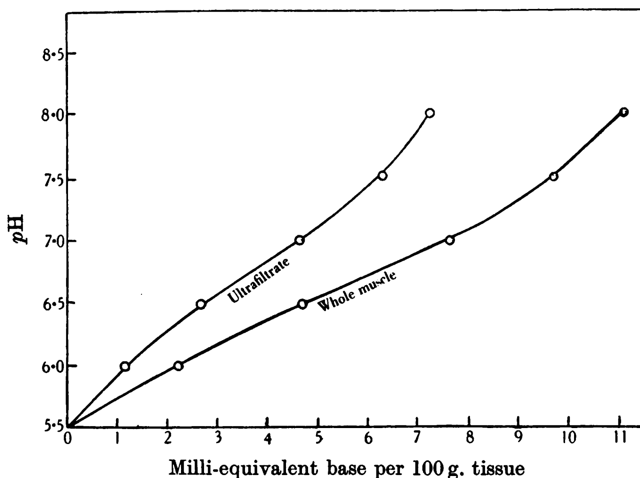

Fig. 1.

Technique

(1) *Titration curves of rigor muscle*

To determine the total buffering power, the muscles are freed from visible fat and connective tissue, finely minced, and a weighed sample of about 20 g. is moistened with a small amount of saline and titrated with 0·1–0·2 *N* HCl or CO_2-free NaOH. *p*H is measured with the glass electrode at room temperature. The most convenient form of electrode for the titration consists of a bulb $\frac{3}{4}$ in. in diameter blown on $\frac{1}{2}$ in. glass electrode tubing. Contact is made internally by means of an Ag-AgCl electrode in 0·1 *N* HCl, and the circuit to the electrometer is completed with a saturated KCl-HgCl half-cell in contact with the brei.

Since equilibrium is attained very slowly in the muscle brei after addition of acid or alkali, it is necessary to ensure that all rapid post-mortem change in the muscle is at an end before beginning the determination. For this purpose, the muscle is kept for 1 hr. at 37° C. in chloroform vapour. In this time the maximum lactic acid concentration is reached and labile phosphorus compounds are converted almost entirely into orthophosphate. In exceptionally well-nourished animals the lactic acid maximum is reached before the muscle glycogen is completely exhausted, and in these cases further formation of acid occurs when

alkali is added. These cases are distinguished by having a pH lower than 5·7, and are dealt with by adding alkali to pH 7·5–8 and incubating for a further period. The curves obtained by titration of the tissue with acid to pH 5·5, and subsequently in the reverse direction with alkali, are then found to follow identical courses.

In constructing such a titration curve, between each addition of alkali or acid it is necessary to allow at least 15, preferably 30 min. for equilibrium to be reached, the brei being well stirred from time to time.

It is essential that no stringy lumps should be present. These are avoided by using a mincer of the "Wedroh masticator" type, which was found to be particularly valuable when only small quantities of material were available.

(2) *Preparation of protein-free extracts*

These have been prepared in three ways:

(*a*) By ultrafiltration of the juice.

(*b*) By aqueous extraction of heat-coagulated muscle.

(*c*) By precipitation with trichloroacetic acid.

Theoretically, since trichloroacetic acid is a strong acid ($pK' < 1$ [Landolt-Börnstein, 1923]) it should have no more effect on the titration of the extract in the range under consideration than the addition of the equivalent amount of neutral salt.[1] This was, in fact, found to be the case. Allowance being made for this effect, no significant differences were observed in the titration curves of the three types of extract, and the last, being the most rapidly prepared, was adopted as a routine method. In preparing the extract care must be taken to restrict dilution. The weighed minced muscle is treated with not more than 50 c.c. of 10 p.c. trichloroacetic acid per 100 g. of tissue, filtered, and a known volume of the filtrate adjusted to pH 5 approximately with a few drops of concentrated CO_2-free NaOH, using methyl red as indicator. After cooling, the titration is continued with standard alkali.

In making such an extract, the soluble buffers are diluted in the proportion $x+y/x$, where x is the volume of water per 100 g. of muscle and y the volume of trichloroacetic acid added per 100 g. of muscle. The titration data is corrected by means of this factor in order to make it applicable to the original muscle. It can usually be assumed, with little error, that the water content of lean mammalian muscle is 75 p.c.

[1] The concentration of sodium trichloroacetate present in the filtrate should, by calculation from Green's [1933] formula, lower the pK_2 of phosphoric acid in 0·03 M solution by 0·2 approx. This it actually does, as could be shown by titration of phosphate solutions with and without the addition of sodium trichloroacetate.

Following the precedents of van Slyke [1922] and Furusawa & Kerridge [1927], the buffering effect may be expressed as the "buffering power" $dB/dp\mathrm{H}$, where dB represents the mg.-equivalents of base required per 100 g. of muscle to produce an increment, $dp\mathrm{H}$, in $p\mathrm{H}$. The latter authors found in minced cat's muscle a sharp maximum in $dB/dp\mathrm{H}$ at $p\mathrm{H}$ 6·5. In most species examined this maximum lies between 6·5 and 7, and is largely due to constituents which appear in the extract (the washed, heat-coagulated protein, suspended in water, gives almost a linear titration curve between $p\mathrm{H}$ 5 and 8). The position of this marked maximum suggests that the buffering of the extract is largely, or entirely, due to the orthophosphate present in it. It has been shown (cf. van Slyke) that the buffering power of a weak acid is at a maximum at a $p\mathrm{H}$ equal to the pK' of the acid, and at the ionic strength of muscle the pK_2' of phosphoric acid is 6·8. If, however, the actual $dB/dp\mathrm{H}$ of the orthophosphate present is calculated from van Slyke's data [1922] or is determined directly by titration of a phosphate solution of equivalent concentration, it is found that a considerable proportion of the buffering still remains to be accounted for. An example of this is given in Table I,

TABLE I. Buffering power of protein-free extract of muscle and of the phosphate therein

Range of $p\mathrm{H}$	$dB/dp\mathrm{H}$ of Whole extract	Phosphate	Unidentified
5·5–6	1·9	1·4	0·5
6–6·5	3·3	2·0	1·3
6·5–7	4·4	2·2	2·2
7–7·5	3·6	1·4	2·2
7·5–8	2·2	0·6	1·6

in which the $dB/dp\mathrm{H}$ of the protein and non-protein fractions of a sample of ox muscle, and of the orthophosphate found therein, are recorded.

A small fraction of the buffering on the more acid side is attributable to lactic acid, which, having $pK'=3{\cdot}9$, has a feeble buffer action at $p\mathrm{H}$ 6, rapidly diminishing at higher $p\mathrm{H}$ values. To satisfy the remaining buffering the substance or substances responsible should also, like phosphoric acid, have a pK' between 6·5 and 7·5 and should be present in the particular instance quoted at a concentration of about 0·04 M. The only known constituent which might conceivably satisfy these requirements is carnosine, which is reported to occur to the extent of about 0·5 p.c. or 0·02 M in the muscles of some species, but no reference to the pK' of this substance (which is a dipeptide of β-alanine and histidine) is to be found in the literature. A sample of the base obtained from

the National Institute of Medical Research was found to have a maximum buffering action at pH 6·9, and therefore fulfils in every particular the requirements for the unidentified buffer substance. In the muscles of other species, particularly of birds, carnosine is accompanied or replaced by anserine, its methyl homologue. This Eggleton[1] has shown to have a $pK'=7{\cdot}1$, so that its buffering range is little different from that of carnosine. Unfortunately, there is not at present an accepted routine method for the determination of anserine.

Proportion of buffering borne by carnosine in muscles of different species

The carnosine, orthophosphate, and total phosphorus[2] contents of the muscles of a number of species have been determined. The method used for carnosine was that of Eggleton & Eggleton [1933], for inorganic and total phosphorus that of King [1932]. In Table II the

TABLE II

		P.c. of muscle				P.c. of buffering due to				
Species	Muscle	Ortho-P	Carno-sine	Lactic acid	Total buffering pH 6–7	Pro-tein	Phos-phate	Carno-sine	Lactic acid	Uniden-tified (possibly anserine)
Horse	Thigh	0·13	0·54	0·94	6·3	45·0	32·0	20·0	1·0	2·0
Ox	Shin	0·14	0·35	0·88	5·3	34·0	44·0	16·0	1·0	5·0
	Thigh	0·15	0·39	0·96	5·65	27·5	46·0	16·0	1·0	9·5
Pig	Psoas	0·17	0·25	0·81	5·8	31·0	47·0	10·5	1·0	10·5
	,,	0·16	0·28	0·90	6·15	37·0	40·0	11·0	1·0	11·0
	,,	0·14	0·27	0·90	5·5	48·0	44·0	11·0	1·0	-4·0
	,,	0·10	0·41	1·20	5·35	45·0	32·0	17·0	1·5	4·5
Sheep	Thigh	0·13	0·14	0·74	5·3	40·0	42·0	6·5	1·0	10·5
	Loin	0·11	0·13	0·68	5·3	45·0	37·0	6·0	1·0	11·0
Dog	Thigh	0·13	0·08	0·75	4·9	35·0	47·0	4·0	1·0	13·0
Rabbit	Long. dorsi	0·15	0·26	1·17	6·2	31·0	40·0	10·0	1·0	18·0
	Thigh	0·14	0·11	0·93	5·75	40·0	40·0	5·0	1·0	14·0
Rat	Mixed	0·19	0·07	0·87	5·3	26·5	55·0	3·0	1·0	14·5
	,,	0·19	0·06	0·74	4·9	26·0	61·0	3·0	1·0	9·0
	,,	0·19	0·07	0·99	5·3	31·0	57·5	3·0	1·0	7·5
Mean of 3 rats	Mixed	0·18	0·06	0·9	4·85	31·0	57·5	3·0	1·0	7·5
Ditto (trained)	,,	0·15	0·05	0·9	5·05	40·0	47·0	2·0	1·0	10·0
Chicken	Leg	0·13	0·05	0·71	4·8	44·0	42·0	2·0	1·0	11·0

results of these determinations are summarized, and the proportion of the buffering of the muscle between pH 6 and 7·5 attributable to protein, phosphate, and carnosine respectively is calculated from this data and included in the table. It is to be noted that muscle taken from a freshly killed animal and treated with chloroform vapour has practically no phosphate other than orthophosphate. The small difference between

[1] Private communication.

[2] Total P was found on the average to be only about 3 p.c. higher than ortho-P. For the sake of brevity the values are omitted from the table.

orthophosphate and total phosphate is probably due to hexose monophosphate, but since the relevant pK' values of the hexose phosphoric esters also lie between 6 and 7 this small amount of esterified phosphate can be regarded as orthophosphate without appreciable error.

The buffering ultimately unaccounted for is at a minimum of 5 p.c. in the horse and ox, and at a maximum of 20 p.c. in the rabbit. The buffering due to carnosine is at a maximum of 20 p.c. in the horse, ox and pig and at a minimum of 2 p.c. in the rat. The values for anserine recorded in the literature are large enough to fill the gap in many cases. Some of these values are collected in Table III, together with some determinations of carnosine in the same species.

TABLE III

Muscle	P.c. carnosine	P.c. anserine	Observer
Horse	0·2–0·5	None	K., K.-A., W.
Ox	0·2–0·6	None	K., K.-A., W.
Pig	0·2–0·4	None	K.-A.
Dog	0·1	0·1	W.
Rabbit	0·05–0·2	0·15	W.
Fowl	0·05–0·15	0·1–0·4	K.-A., W.

K. = Kuen [1927], K.-A. = Kapeller-Adler and Haas [1934], W. = Wolff & Wilson [1935].

Variation in buffering of muscle

As the figures in Table II show, there is a fairly wide variation between animals of different species, the lowest recorded being 4·8 for the rat, the highest 6·3 for the horse. These differences are of considerable practical importance, for they are large enough to determine the fate of a carcase. In the case, for instance, of two muscles each containing 0·7 p.c. of glycogen, in which 8 milliequivalents p.c. of lactic acid would be produced, the muscle with a buffering power of 4·8 would reach pH 5·9, whilst that with a buffering power of 6·2 would reach only pH 6·3 and would be in danger of undergoing rapid putrefaction or of becoming "bone tainted". Unfortunately there does not seem to be any simple means of lowering the buffering power of muscle without at the same time lowering its glycogen reserves. The highest gross variation is in the phosphate fraction (from 1·6 to 3·0) corresponding to a variation of 0·1–0·2 p.c. of phosphorus. Individuals of any one species appear to vary within rather narrower limits. A large number of albino rats have been examined, and irrespective of age and treatment the buffering from pH 6 to 7 fell within the limits of 4·8–5·3.

With the possible exception of carnosine and anserine, none of the buffer constituents appears to be readily variable at the will of the

experimenter. Hunter [1925] showed that a short period of starvation caused a rapid loss of carnosine from the muscles in cats, and that it was readily restored by feeding with meat. However, starvation of longer duration than is already customary could hardly be recommended as a means to this end. Meat animals are, as a routine measure, starved for about 24 hr. before slaughter, yet the muscles of oxen and pigs so treated still contain the high concentration of carnosine recorded in Table II.

The position of carnosine and anserine in muscle physiology is difficult to interpret. No function has hitherto been allotted to these bodies, but it could certainly not be stated with any confidence that their role is specifically that of muscle buffers, for they vary in concentration both individually and collectively within such wide limits. Yet their buffering action cannot be ignored, for it may amount to 25 p.c. of the buffering at *p*H 7 of rigor muscle, or as much as 40 p.c. of the buffering of living muscle, in which most of the phosphate is esterified and in the form of substances which buffer comparatively weakly between *p*H 7 and 7·5. The *pK′* of phosphocreatine is 4·5, of hexose monophosphate 6·1 [Meyerhof, 1930]. Adenyl pyrophosphate with three phosphorus atoms in its molecule has only one hydrogen ion dissociating in this range [Lohmann, 1933].

The total buffering of the phosphate system in resting muscle works out at less than a quarter of that in rigor muscle. Taking, for instance, the distribution of phosphorus in resting aerobic frog's muscle as given by Eggleton & Eggleton [1929], the following estimate of the buffering at *p*H 7, in terms of the buffering of orthophosphate, can be made.

	P.c. of total P	Approximate buffering as orthophosphate
Orthophosphate	13–15	14
Phosphagen	49–53	Nil
Adenylpyrophosphate	31–32	10
Hexosephosphate	2–4	2
	Total	26

If similar values can be assumed for mammalian muscle with as much as 0·6 p.c. of carnosine it is evident that the buffering due to carnosine would be more than double that due to phosphates, and equal to that due to protein.

Buffering due to bicarbonate in living muscle

Fenn [1928] and Irving *et al.* [1932] have shown that the buffering due to bicarbonate *as such* at the *p*H of living muscle (estimated at 7·0–7·2) is quite small. In the frog, Fenn estimates that of a total

*dB/dp*H of 2·0 only 0·4 is attributable to HCO'_3; in the dog, Irving *et al.* find 0·3 of a total of about 4 attributable to carbonates. In virtue of *escape of* CO_2 from bicarbonate, the buffering power is of higher order, and represents at least half the total buffering power of the muscle. It should be noted, however, that buffering at this intensity can be maintained for only so long as bicarbonate is available, and calculation from the data of these authors shows that the mean content of 20 c.c. of CO_2 per 100 g. of resting muscle corresponds to no more than 0·01 *M* bicarbonate sufficient to accommodate only 0·09 p.c. of lactic acid. Fatigued muscle, according to Meyerhof [1922] contains practically no bound CO_2; as would be expected, the buffering by escape of CO_2 is exhausted by only a fraction of the lactic acid produced during prolonged contraction. While, therefore, the bicarbonate buffering is a powerful factor in stabilizing *p*H during the early (almost anærobic) stages of severe exercise, its effect rapidly weakens and, if lactic acid continues to be produced, eventually vanishes. The buffering then rests entirely on the protein, carnosine-anserine and phosphate fractions of the muscle.

Summary

60–75 p.c. of the buffering of mammalian skeletal muscle in rigor between *p*H 7·5 and 5·5 is due to non-protein substances. Orthophosphate accounts for about one-half of this non-protein buffering. The remainder is largely due to carnosine and anserine, which buffer strongly in this range with a maximum of buffering at about *p*H 7.

The possible importance of carnosine and anserine as buffers in living muscle is discussed.

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