Spectrophotometric Studies on the pH of Frog Skeletal Muscle

*pH Change during and after Contractile Activity*

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**ABSTRACT** The spectral characteristics of the pH-sensitive dyes neutral red (NR) and bromcresol purple (BCP) were utilized for studies of the changing intracellular pH (pHᵢ) of sartorius muscles from Rana pipiens, both during the course of an isometric twitch and during recovery metabolism subsequent to a train of twitches. The information from the two dissimilar dyes correlated to confirm the methodology. Neither the fast realkalinization observed during a twitch nor the slow alkalizing phase of recovery metabolism was affected in an obvious manner when phosphocreatine (PC) hydrolysis was blocked by 1-fluoro-2,4-dinitrobenzene (FDNB). Iodoacetic acid (IAA) did inhibit the slow acidic phase of recovery metabolism. The conclusion is made that alkalizing reactions other than PC breakdown must be considered as operative at these levels of activity. Hypertonic solutions altered twitch tension and time course without altering the pHᵢ shifts observed until approximately 75% of the twitch amplitude was abolished. Multiple effects of hypertonic solutions as the muscles approach tonic equilibrium are proposed.

**INTRODUCTION**

A variety of metabolic products have been demonstrated to arise from the energy-utilizing reactions involved both directly and indirectly with muscle contraction. Many of these have the effect of altering the intracellular pH (pHᵢ) as they are formed or degraded (Dubuisson, 1939; Mommaerts, 1950; Nishimura et al., 1962; Novotny, 1968; Mommaerts, 1969; Adler, 1972). By continuously monitoring the changes in pHᵢ brought about by these active processes it should be possible to examine the relative kinetics of the reactions in an intact preparation.

The ability to continuously monitor qualitative changes of pHᵢ spectrophotometrically, both at the level of the isometric twitch and during recovery metabolism, has previously been demonstrated for toad (Jöbsis, 1967) and frog (Jöbsis, 1963) sartorius muscles. It was our intention in undertaking the present experiments to expand on these initial results, both examining the validity of the
methodology as well as utilizing it to further elucidate the details of the energy metabolism accompanying contraction.

A primary concern has been to prove that the signal-averaged optical data obtained during the course of single isometric twitches were not merely an artifact resulting from sample movement in the light path. We therefore present results obtained with two dissimilar pH-sensitive dyes, neutral red (NR) and brom cresol purple (BCP), which undergo spectral shifts of opposite polarity when subjected to changes in pH. These data serve to corroborate our view that what we have measured reflects or is in fact a change in pH.

A portion of this investigation involved the use of the metabolic inhibitors 1-fluoro-2,4-dinitrobenzene (FDNB) and iodoacetic acid (IAA) to resolve the events responsible for the observed pH shifts. The former compound blocks the Lohman reaction at the level of creatine phosphotransferase (Cain and Davies, 1962; Dydynska and Wilkie, 1966), while the latter compound blocks glycolysis at the level of glyceraldehyde phosphate dehydrogenase (cf. Jöbsis, 1964). Data were also obtained concerning the nature of the inhibition of contraction by hypertonic solutions, where separation of E-C coupling and twitch generation was observed.

METHODS

Muscle Preparation

The preparation utilized in these experiments was the sartorius muscle from the frog Rana pipiens. The animals ranged in size from 3½-4 inches in length, nose to anus. The standard Ringer's solution consisted of 113 mM NaCl, 5 mM KCl, 2 mM CaCl₂, and 2 mM Na₂PO₄, adjusted to pH 7.0. For overnight storage of tissue the temperature was lowered to less than 5°C, 10 mM pyruvic acid included, and moist 100% O₂ bubbled through.

The frogs were killed by decapitation and the legs cleared of blood by perfusion through the descending aorta with standard Ringer's. Rest length (Lₒ) was determined before excision by measuring the distance from the pubic symphysis to the point where each muscle was tied with button thread (cotton #8) at the tendon inserting into the knee (tendon end).

Hypertonic solutions were formulated by adding either sucrose or D-mannitol to the standard Ringer's so that the total molar concentration of ions plus molecules in solution was at the desired level. For example, 2 × R Ringer's consisted of standard Ringer's plus a molar concentration of sugar equal to the total molar concentration of ions in solution in the standard Ringer's. The indicators were administered by soaking the tissue in the dye solution for variable periods of time (see text).

Dual Wavelength Spectrophotometry

pH transients were followed by monitoring the relative changes in amplitude of one of the absorption peaks of a pH-sensitive dye equilibrated into the muscle. The instrument utilized for these measurements was a dual-wavelength (double-beam) spectrophotometer basically of the type described by Chance (1957). Optical chopping was accomplished by revolving a slotted wheel placed at an angle between the outputs of the two monochromators and a fixed half-silvered mirror.

The beams were focused onto the polished face of a light-conducting fiber placed in the image plane of the gratings. The other end of the fiber bundle was incorporated into
the mounting platform of the tissue chamber (Fig. 1) and polished flush with the surface in contact with the muscle. Here the fibers were arranged in a rectangle to provide an optical window 1.5 x 9.5 mm.

The muscle holder in Fig. 1 was designed to minimize artifacts due to movement of the muscle during contraction. When threaded as shown, the tissue was held firmly in place against the supporting platform, while allowing tension to be recorded at the tendon end. Muscle length was either set by measurement at approximately 110% of its length in the body or simply mounted with 10-20 g of initial rest tension.

**Figure 1.** Diagram of the muscle chamber attached to the dual wavelength spectrophotometer. The muscle is held firmly in place over the mounting platform, the polished face of which contains the insertion of one end of an optical light fiber assembly. The other end inserts into a polished surface to which the grating image from each monochromator is focused. Stimulation is delivered by means of two parallel stainless steel electrodes.

Light transmitted through the tissue impinged upon the face of an RCA 6342-A end-window photomultiplier tube (PM) (RCA Corp., Camden, N.J.) positioned in front of the chamber 1.5 cm from the muscle surface. The 2-inch photocathode subtended an angle of approximately 120°. The voltages of the selected middle portions of each illumination half cycle provided the inputs to a differencing amplifier, the output of which was an expression of the difference in absorption by the tissue of the two alternating light beams. When chopping at 1 kHz, a time constant of 2 ms was achieved.

The spectral band width of the reference monochromator was fixed at 9.9 nm, while that of the sample monochromator varied, but averaged around 1.6 nm. Zero percent and one hundred percent absorption difference between transmitted sample and refer-
ence beams were determined by appropriate adjustments of the sample monochromator's vertical and horizontal exit slits.

The double-beam method is used to suppress the optical artifacts produced during contractile activity. Since scattering increases with decreasing wavelength, the dual wavelength technique cannot completely eliminate the difference in light scattering between the sample and reference beams. This becomes a sizable error when scattering predominates over true OD changes as was the case in these experiments. However, by proper manipulation of recorded signals from reference wavelengths shorter and longer than the sample wavelength (in these instances corresponding to the high wavelength absorption peak of whichever dye is used: 530 nm for NR and 590 nm for BCP), it is possible to obtain almost complete cancellation of this remaining artifact. For example, if the reference monochromator is set at a wavelength 25 nm longer than that of the sample monochromator, the optical signal received during the contraction of a dye-equilibrated muscle will be a mixture of some extra light scattering plus the signal produced by changes in dye absorption. If the reference beam is then set 25 nm to the opposite side of the dye's absorption peak and the same activity initiated, the new signal will consist of a mixture of dye absorption changes and a scattering artifact which is of opposite sign as before. Addition of these two sets of signals will tend to cancel the light-scattering artifact, whereas the dye-produced signal will be enhanced (Jobsis and O'Connor, 1966). Complete cancellation could be achieved by varying the reference wavelengths for each individual preparation, but the slight improvements in signal did not warrant this time-consuming control study. Furthermore, alteration by the dyes of the absorption characteristics of the preparations also changes the effects of light scattering, rendering predyed controls of limited value.

The signal-to-noise ratio for individual twitches was improved by means of a computer for average transients (Nuclear-Chicago 7100, Nuclear-Chicago Corp., Des Plaines, Ill.). For NR a reasonable signal-to-noise ratio for the optical signal generated by a twitch was achieved by averaging the outputs of 20 twitches. With BCP an average of 10 sweeps was used.

Considerable variability was found to exist between individual preparations in the light-scattering artifact accompanying and after contractions. However, this was much less the case in the wavelength-specific dye signal after subtraction of the signal at the reference wavelengths. Also the ratio of the dye-specific event to the combined dye-plus-scattering signal depended on the dye used, being smaller for NR than for BCP. Thus the ratio of the computer-extracted signal peak to the combined signal-plus-artifact peak is variable and related to the dye used. For NR this ratio was between 0.2 and 0.5, while for BCP it was between 0.9 and 1.0. In all cases the amplitude of the random high frequency noise in the system fluctuated between 0.1 and 0.2%. Slow changes in the absorption peak were monitored by observing slow changes in the absorption difference between the peak and one reference wavelength.

Experimental Procedures

The dye-equilibrated muscle was mounted in the chamber and the proper level of supramaximal stimulation determined. The chamber solution (approximately 100 ml) was changed periodically by draining and refilling with fresh Ringer's, except in experiments on the time course of washout of BCP from the muscle. Here a continuous flow of solution into the chamber was provided by gravity feed, the overflow being removed by aspiration.

Initially the muscle was quickly rinsed to remove surface dye, then soaked in Ringer's for approximately 5 min before new Ringer's was added and the experiment begun. In
experiments where the chamber was drained during stimulation, the first two rinsing steps were followed then the chamber was drained. Under these conditions a film of solution between the electrodes across the muscle maintained electrical contact for supramaximal stimulation.

Biochemical Assays
Analyses on PC breakdown were carried out on muscle pairs from the same animal, stored overnight in 10 mM pyruvate Ringer's at 3°C with moist oxygen bubbling. The bases of individual muscles were clamped to an L-shaped metal rod (an "L") which was affixed to a Plexiglas block. The muscle was set at its rest length and was then lowered into the appropriate Ringer's solution for a total of 1 h at room temperature (23°C) with moist oxygen bubbling. When FDNB was used to poison the muscle it was added during the last 30-45 min. Stimulation was supramaximal, delivered through the mounting L and another electrode lowered into the Ringer's. The muscle was then raised out of the solution and frozen quickly in petroleum ether cooled in liquid nitrogen while still mounted at rest length.

Perchloric acid extractions were carried out according to the method described by Dydynska and Wilkie (1966). Assays for creatine and creatine phosphate were done according to Ennor (1957).

RESULTS
Washout of Dye
Washout experiments were undertaken with BCP. However, no such experiments were done with NR, since only a single rapid washout phase of short duration was ever observed with this dye.

Fig. 2 demonstrates the biphasic nature of the washout of BCP from these muscles: an initial fast phase with a half-time of approximately 10 min and a slow phase with a half time of 56 min. Since the muscles were initially rinsed before washout to eliminate surface dye, observations of the earliest time course were precluded. The half time of the rapid phase may therefore have been slightly shorter: on the order of 5-7 min. Extrapolation of the slow washout phase back to zero time confirms that even when the dye is not being washed out, dye in this phase is responsible for over 90% of our recorded signals.

Intracellular pH Transients, NR Versus BCP
Fig. 3A contains the computer readouts for the first 20 twitches of a muscle equilibrated overnight in 0.025 mM NR and mounted in the chamber with approximately 10 g initial tension. The muscle was rinsed once with dye-free Ringer's, then immersed in fresh Ringer's solution.

The lower trace in Fig. 3A is the computer-averaged optical readout for the absorption changes for 10 twitches with the sample monochromator set at 530 nm and the reference monochromator set at 505 nm, plus 10 with the reference monochromator switched to 555 nm. A slight delay occurs between the onset of contraction and the onset of acidification, the latter shift represented by a shift upward in the optical trace, the acid shift reaching its peak at approximately the same time as the tension. Although it is not well defined here, there often occurs
an overshoot in the basic direction before recovery towards initial pH₄ at the end of a twitch. The traces in Figs. 6 and 7, for example, show clear overshoots which are not related to features of the myogram. The nature of the reactions giving rise to this effect are presently unknown. A marked early undershoot is occasionally observed in NR-dyed muscles (see Fig. 8A) which has been determined to be related to a movement artifact resulting from the muscle-shifting position in the beam path. Where this shift was too exaggerated or could not be eliminated by stretching or remounting the tissue, the preparation was discarded. This eliminated approximately 1 in 10 muscles.

Fig. 3B contains the same data for another muscle from the same batch of frogs equilibrated overnight with 0.1 mM BCP. Exactly the same procedures were used except that the sample monochromator was set at 590 nm and the reference alternately at 565 and 615 nm.

The relative time courses of the tension peak (upper trace in Fig. 3B) and the acid peak (this time represented by a downward deflection of the lower trace) are similar to those observed with NR. The form of the realkalinization process is, however, different; the half time of return to the base line occurring after the tension has reached nearly 80% recovery. A simple exponential does not appear to adequately describe this rise in pH. The time course of the total pH₄ change is typical, although the time to peak may shift by a few milliseconds in either direction, depending upon the muscle used.

A difference in kinetic results is to be expected for the two dyes, since each appears to be contained in a different compartment of the muscle (Macdonald et al.).¹ The faster time course of recovery for NR is reasonable, since it appears to

¹ Spectrophotometric measurements of metabolically induced pH changes in frog skeletal muscle. Manuscript submitted for publication.
equilibrate inside the cells, whereas the BCP appears to reside in some outside compartment. Regardless of the kinetic differences imparted by selective compartmentalization, qualitative comparisons of the results obtained with the two dyes are valid, since the pH_i shifts exhibit the same general form. Either dye may thus be used to examine relative shifts in pH_i elicited by contraction.

These results serve as a confirmation of the method's validity. If the shifts in the optical records were caused by a movement artifact, the change in amplitude

![Figure 3](image_url)

**Figure 3.** Relationships between twitch tension and the fast pH_i shift for muscles contracting isometrically, NR vs. BCP. Both A and B are readouts for 20 twitches averaged in the computer. In each the upper record is the tension trace and the lower record is the optical trace. Muscle A was equilibrated overnight with 0.025 mM NR and twitched 10 times at 530-555 nm plus 10 times at 530-505 nm. Muscle B was equilibrated overnight with 0.1 mM BCP and twitched 10 times at 590-565 nm plus 10 times at 590-615 nm. For NR acidification leads to an upward deflection. Both results tend to confirm one another. The small verticle line placed over each tension trace 12.5 ms from the start of the scans is to mark the stimulus.

The optical transients observed were a true index of changing pH_i, then the signals should be of opposite sign, but similar in the shifts they depict. This proved to be the case.

**Effects of Metabolic Inhibitors**

The simplest interpretation of the pH_i changes accompanying an individual twitch is that the initial drop in pH_i is due to ATP breakdown while the subsequent increase is due to rephosphorylation of ADP by PC. A net alkaline
change is expected due to the release of creatine (Mommaerts, 1969), a relatively strong base. Indeed, the existence of a cumulative basic shift resulting from extended twitch activity has been known for years (Dubuisson, 1939; Jobis, 1963).

A series of experiments was carried out using FDNB to block creatine phosphoryltransferase (Cain and Davies, 1962; Infante and Davies, 1962) in order to directly test the hypothesis that the slow alkalinization depicted in Fig. 4B is in fact due to PC breakdown. Single isometric Twitches were also studied in the hope that the origin of the fast basic shift would be ascertained.

The FDNB experiments were carried out on pairs of frog sartorius muscles excised from the animals and generally stored overnight in oxygenated Ringer's at 2-3°C with 10 mM pyruvate; although in several instances storage time was extended over a period encompassing as many as three nights. For the experiment depicted in Fig. 4 the muscle was stored in pyruvate-BCP Ringer's for three nights and mounted with approximately 20 g of initial rest tension.

Fig. 4A and B demonstrate the normal fast and slow pH₁ shifts accompanying, respectively, individual isometric twitch contractions and a series of 40 Twitches

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**Figure 4.** Effect of FDNB on the twitch tension and resultant fast and slow pH₁ shifts. This muscle was equilibrated overnight in 0.1 mM BCP. In all cases decreasing pH₁ is reflected by downward shifts of the optical traces. For A, C, and E the top trace of each pair of records represents tension, the bottom ΔpH₁. The scales in A correspond to 50 ms, 15 g, and 0.5% and apply for C and E. All data were collected with a drained chamber. A, C, and E are the average of 10 sweeps on the computer, five at 590-615 nm plus five at 590-565 nm. A and B are controls. C then D were taken after 25 min in 0.3 mM FDNB Ringer's. E was taken after the train in D.
at a frequency of 1/2s. After a recovery period of 30 min in the BCP Ringer's, a new Ringer's was added which contained, in addition, 0.3 mM FDNB. After 25 min, fresh Ringer's without dye was substituted for 5 min and the chamber drained. The same series of isometric twitches was then repeated.

In Fig. 4C the amplitude of the averaged twitch tension is affected by the FDNB. The form and time course of the accompanying optical trace are minimally affected. A set of 40 stimuli was again delivered (see Fig. 4D), and there was a marked sequential decrease in twitch amplitude which is characteristic of FDNB-poisoned muscles (Dydknska and Wilkie, 1966). However, the slow alkalization observed in the normal case is still present, as is the slower reacidification.

Fig. 4E contains another set of averaged traces from the computer for 10 stimuli applied immediately after the slow series just mentioned. Twitch tension amplitude is decreased even further, although the time course is about the same as before, while the realcalizing phase of the pH shift is still present.

In order to check whether the creatine phosphoryltransferase reaction was effectively blocked, a small series of muscle pairs was analyzed for C and PC. The results of this analysis are shown in Table I. Apparently FDNB did block the transferase reaction. This is most easily seen from the calculated C/PC ratio for each muscle.

A series of experiments was undertaken in order to test the effectiveness of treatment of the muscle preparations with IAA. For the experiment depicted in Fig. 5 one member of a pair of muscles, stored in the BCP solution as previously described, was mounted with approximately 20 g of initial tension. The usual 5-min clear Ringer's rinse was then applied and the chamber drained. The control in Fig. 5A shows the normal slow alkalization followed by acidification elicited by a train of twitches. Recovery was allowed for 30 min in 0.1 mM BCP plus 10 mM pyruvate Ringer's. The same solution minus the pyruvate but with 1.0 mM IAA was then added for 45 min. After the 5-min clear Ringer's wash the chamber was again drained and the stimulation repeated. In Fig. 5B the late acidification is completely abolished, leaving a very much enhanced latent alkalization.
From these results it is concluded that in the absence of PC splitting alkalizing reactions still occur with twitch activity, as expressed during both the rapid and slow phases of pH rise change. Other energetic reactions involved in early energy metabolism in muscle apparently produce significant quantities of basic compounds. The myokinase reaction coupled with deamination of AMP to IMP is one candidate for such an effect (see Kushmerick and Davies, 1969).

**Effects of Hypertonicity**

With the discovery that hypertonic solutions abolish the twitch tension of a muscle fiber without abolishing membrane excitability (Hodgkin and Horowicz, 1957), the question arose as to the site of action of this effect. The following experiments were undertaken in order to examine this point.

In one series of experiments muscles were utilized that were soaked overnight in NR-Ringer's at 2-3°C with moist oxygen bubbling. Each muscle was mounted at approximately 110% of its length in the frog before excision. After a 5-min rinse with clear Ringer's the chamber was flushed and new clear Ringer's added. The experiments were run with a Ringer's-filled chamber, rather than a drained one. Experiments were done utilizing either sucrose or D-mannitol to achieve hypertonicity.

Fig. 6A shows the normal tension and optical traces for one muscle, while Fig. 6B shows another set of data 8 min after application of 1.5 × R sucrose Ringer's. Each set of data is composed of 20 twitches averaged by the computer. Whereas
the isometric tension trace is considerably altered by the hypertonicity of the solution, the optical trace is virtually unchanged in its rise to peak and return towards rest level. Aside from a decrease in magnitude, the time course of the myogram is delayed somewhat whereas the time course of the pHᵢ shift is unaltered except for a slight lengthening of the recovery phase.

Several concentrations of d-mannitol were applied to different muscles. Fig. 7 demonstrates the effects on twitch tension and changing pHᵢ of 1.5 × R d-mannitol Ringer’s. Fig. 7A shows the normal tension versus pHᵢ, while Fig. 7B shows the information 8 min after introduction of the hypertonic solution. There is a significant shift in the time course of the twitch towards a longer time period, while the amplitude of the optical trace is only slightly affected. These results are supported by similar experiments where no decline in the optical peak was observed. Normal Ringer’s for 12 min initiated a slight recovery of twitch tension height, and a normal-size optical trace can be seen (Fig. 7C). Resoaking for 10 min in the d-mannitol Ringer’s led to an even better separation of the optical and tension peaks (Fig. 7D).

It appears to be a characteristic response to hypertonic solutions that a
diminution of twitch tension below one-quarter the control value is accompanied by a decrease in the amplitude of the optical trace. This is demonstrated by the experiment utilizing $2 \times R$ d-mannitol Ringer's shown in Fig. 8. Fig. 8A shows the control tension and optical data for 20 twitches. After 6 min in $2 \times R$ Ringer's the initial motion artifact disappears, and a more normal pH$_1$ shift is seen (Fig. 8B), while the amplitude of the twitch is decreased, and the time course is slowed down. Fig. 8C shows that the separation between pH$_1$ shift and tension is increased upon prolonged exposure (up to 21 min) to the hypertonic solution. Forty minutes of exposure (Fig. 8D) results in further separation of pH$_1$ and tension kinetics, but now the optical trace shows a decrease in amplitude.

Neither a slow resting tension increase nor pH$_1$ change was observed during experiments in which up to $2 \times R$ Ringer's solutions were used with these muscles.

Similar experiments with BCP-dyed muscles proved to be less reliable quantitatively. Although the kinetics of the pH$_1$ shift were little affected by increased tonicity, a significant decrease in its amplitude was observed. Sometimes the amplitude decrease was proportionately the same as the drop in twitch tension, sometimes more and sometimes less. This instability is to be expected, considering the susceptibility of the dye to washout and its apparent equilibration into predominantly extracellular compartments (Macdonald et al.). The pH$_1$-related optical shifts observed with NR are considered to be reliable in a quantitative sense, since this dye exhibits much greater stability in the preparation than is the case with BCP.

The disparity between the effects of hypertonic solution on muscle twitch and on the accompanying pH$_1$ shifts, especially evident in the more quantitative NR results, plus the delayed decline in the optical signal, indicate that more than one mechanism is likely involved in the progressive effects which hypertonic solutions exert on muscles.

**DISCUSSION**

The metabolic reactions known to accompany contraction (for reviews see Jöbsis, 1969; Mommaerts, 1969; Woledge, 1971) have previously been fitted into the scheme outlined by Dubuisson (1939), where an acidification ascribed to ATP hydrolysis is followed by a net alkalinization attributed to PC breakdown, which is then followed by an iodoacetic acid-sensitive, slow acid shift attributed to lactic acid production. This same pattern of shifting pH$_1$ has been noted in this laboratory using BCP in association with toad sartorius muscles (Jöbsis, 1963) and by us in frog sartorius muscles. However, doubt is cast upon the metabolic origin of the slow alkalinization, previously attributed to PC breakdown, by the results shown in Fig. 4, so that one or more alternate or additional reactions must be postulated to explain this slow basic shift.

It has been known for some time that ammonia production accompanies and is proportional to muscular work (for review see Lowenstein, 1972). Various workers using muscle extracts and monitoring pH electrometrically have found that ATP splitting causes a decrease in pH, which is followed by a pH increase due to ammonia formation from deamination of AMP produced from ADP by
FIGURE 7. The effects of 1.5 × R d-mannitol Ringer's on the twitch tension and rapid pH$_1$ shift recorded with NR. The same procedure for averaging the data on the computer was used here as in Fig. 6. Again, there occurs a separation between the effects of the increased tonicity on tension production and the reactions responsible for the pH$_1$ transient. A is the normal case, while B occurs 8 min after exposure to the hypertonic Ringer's. C demonstrates some recovery of twitch amplitude when the muscle is exposed to normal Ringer's for 12 min. D is the data obtained from subsequent reintroduction of the d-mannitol Ringer's for an additional 10 min. This leads to an even larger separation of the two signals.

FIGURE 8. The effects of 2.0 × R d-mannitol Ringer's on the twitch tension and rapid pH$_1$ shift recorded with NR. These data were extracted in the same manner as previously outlined for NR-equilibrated muscles. A pertains to the normal state of the muscle in the standard Ringer's. The optical trace appears to have a large movement artifact, which disappears in B after 6 min in the hypertonic Ringer's. The primary importance of the data shown here is that they are excellent examples of how the tension time course can be slowed down to a very great extent by these conditions without affecting the time course of the pH$_1$ shift. Even when the amplitude of the pH$_1$ signal begins to decline its time course remains virtually unaltered.
the myokinase reaction (Herniö and Saris, 1967; Novotny, 1968). Furthermore, it has been shown in frog muscles that although PC breakdown is blocked by FDNB (Infante and Davies, 1962) the myokinase is not blocked (Cain and Davies, 1962).

Recently the existence of a purine nucleotide cycle in rat skeletal muscle extracts was demonstrated (Tornheim and Lowenstein, 1972). Such a system may account for the basic shift observed and could be envisioned as an alternate pathway for ATP generation as the myokinase reaction is shifted in favor of ATP production (Lowenstein and Tornheim, 1971) when other energy-producing pathways are saturated or blocked.

At the level of the individual twitch an overshoot in the basic direction is observed with NR (see Fig. 7A), which is consistent with the net alkalinization expected from ATP-PC breakdown (Mommaerts, 1969). However, it appears that the fast realkalizing shift recorded at this level may be the result of chemical events other than PC breakdown (Fig. 4). Although objections have arisen to refute the production of hypoxanthine nucleotides at this level of activity (Cain et al., 1963) they are overcome by Tornheim and Lowenstein’s (1972) demonstration of a closed-cycle reaction in which no net increase of IMP is necessary for the deamination of AMP. In fact, Infante and Davies (1962) observed AMP formation in muscles frozen at the peaks of their contractions, and earlier spectrophotometric evidence was claimed to indicate the production of hypoxanthine nucleotides at the level of the twitch in frog semitendinosus muscle (Wajzer et al., 1956).

The possibility therefore exists that it is this ammonia production which is responsible for the observed basic shift during isometric twitch relaxation, at least under conditions of FDNB poisoning. It cannot be ruled out that under normal conditions the reserve high energy phosphate stores inherent in both the PC and purine nucleotide cycle work within the framework of a reciprocal relationship. However, if this were the case we would perhaps expect more of a difference in effect on the kinetics of the pH shift than was observed when the muscles were poisoned.

When muscle fibers are exposed to hypertonic solutions their volumes decrease (Blinks, 1965), and electron micrographs show a shrinkage of the A-band tubules as well as a packing together of the filaments (Dydynska and Wilkie, 1963). Coupled with these effects is a decrease in active tension with increased tonicity (Howarth, 1958), although stretch experiments demonstrate a normal ability of the muscle to bear its full isometric tension soon after the stimulus. In terms of presently accepted concepts this means that the “activation process” still occurs. This, coupled with Smith’s observations (1975) that moderately strong hypertonic solutions exert no effects on activation heat, indicate that the effects of hypertonicity occur after E-C coupling.

In our experiments, during the first 75% of the decline in twitch amplitude due to hypertonic solutions, separation is achieved between the generation of twitch tension and the chemical reactions involved, the former becoming markedly retarded whereas the latter show very little change in time course. This fits with a mechanical damping of the contraction due to binding of the filaments.
The later effects, after a 75% decline has been exceeded, correspond either to
direct interference in the reactions by increased ionic strength (April et al., 1968;
April and Reuben, 1968), interference with E-C coupling (Lännergren and
Noth, 1972, 1973), or both (Gordon and Godt, 1970; Homsher et al., 1974).

We cannot arrive at any firm conclusions regarding the relationship between
our results and those of Homsher et al. (1974), where increased tonicity was
observed to interfere with activation heat production. We do not know what
percentage of our pH\textsubscript{i} shift is due to those mechanisms involved with calcium
release and reuptake, although we do suspect that these processes provide a
minor contribution. Our measurements, unlike theirs, were generally made
before the effects of increased tonicity were maximal; that is, before tonic
equilibrium was attained. It is therefore likely that at least a portion of the late
decline in our optical signal (see Fig. 8D) is due to direct interference with the E-
C coupling mechanism.

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