Spectrophotometric Studies on Intact Muscle

II. *Recovery from contractile activity*

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ABSTRACT The kinetics of the mitochondrial respiratory chain of intact muscle and the concomitant changes of the intercellular pH were investigated. Addition of lactate and pyruvate under resting conditions produces reductions of DPN and cytochrome *b*, and, occasionally, of cytochrome *c* and flavoprotein. Succinate gives similar but smaller changes. In recently excised muscles moderate contractile activity produces a reduction of cytochrome *c* and oxidations of DPNH, cytochrome *b*, and sometimes of the flavoproteins. Tetanic contractions and larger numbers of twitches produce reductions of DPN and of cytochromes *b* and *c*. In sartorii of the tropical toad, stored for approximately 2 days at 0–3°C, contractile activity always gives rise to long lasting oxidations of DPNH and cytochrome *b*. Addition of pyruvate or lactate shortens these oxidation cycles with a concomitant reduction of cytochrome *c*. These responses to contractions agree with those of mitochondria isolated from leg muscles of the toad upon the addition of ADP. Apparently the mitochondria in resting, excised muscles are not supplied with an excess of substrate. Measurements on the intercellular pH showed that even limited activity (< 5 twitches) initiates glycolysis. The primary control of respiration resides, nevertheless, in the ADP concentration, rather than in the levels of substrate or inorganic phosphate. The results are quantitatively consistent with the view that ATP is the primary energy donor for muscular contraction.

INTRODUCTION

In the preceding paper (Jöbsis, 1963) the occurrence of the members of the respiratory chain in various intact muscles was established. Their concentration and the oxidation-reduction levels in the resting muscles were determined with the exception of diphosphopyridine nucleotide (DPN) and presumably of some fractions of the flavoproteins. In this article, the changes in the steady-state levels of these components upon the disturbance of the resting state by contractile activity and during the subsequent return to resting conditions will be examined.

Previous work on isolated mitochondria has established the dependence of

the most rapid respiration on adequate supplies of substrate, inorganic phosphate and phosphate acceptor (adenosine diphosphate, ADP), or an uncoupling agent. Subsequently, Chance and Williams (1955) measured the dependence of the steady-state oxidation-reduction levels of the respiratory carriers on the concentrations of these agents and found characteristic differences. Thereafter, Connelly and Chance (1954) found that the DPNH of excised frog sartorii underwent a temporary oxidation as a result of a series of single twitches. This last work showed that the well known increase in the rate of O_2 uptake after contractile activity (Meyerhof and Schulz, 1927; D. K. Hill, 1940) is reflected in the steady-state oxidation-reduction level of at least this member of the respiratory chain.

The studies presented here are an extension of this approach. They were launched with the general intent of clarifying the relations between contractile activity and recovery metabolism. It was hoped that the results would afford a basis for a discussion of the question whether ADP can be considered as the product of a primary reaction yielding energy for the contractile activity.

The observations of Chance and Williams (1955) form the background for the present ones. These investigators found, with isolated mitochondria suspended in a suitable reaction medium, that several members of the respiratory chain show characteristic steady-state oxidation-reduction levels in the various, definable states of metabolic activity. After isolation from the tissue the particles contain a limited amount of endogenous substrate, but respiration is slow because of a lack of a high energy phosphate acceptor (state 1). In this state DPN, the flavoproteins, cytochrome *b*, and cytochrome *c* are partially reduced. Upon addition of ADP, the respiration speeds up momentarily until the substrate is exhausted; O_2 consumption falls to zero (state 2), and the respiratory carriers become completely oxidized. Addition of a suitable substrate at this moment initiates a rapid respiratory rate (state 3) until all the ADP has been removed, at which moment the respiration falls to a low level (state 4). In this condition DPN is approximately 100 per cent reduced, the flavoproteins and cytochrome *b* around 40 per cent reduced, cytochrome *c* 10 to 15 per cent reduced, while cytochromes *a* and a_3 are better than 99 per cent oxidized. Addition of ADP at this time produces again an increase in the respiratory rate with concomitant changes in the level of reduction of the components of the chain (state 3). DPNH becomes more oxidized; about 50 per cent remains reduced. The flavoproteins and cytochrome *b* also show a decreased reduction, down to 25 and 18 per cent, respectively. Cytochrome *c* can respond either with an oxidation or a reduction depending on the origin of the mitochondria and, perhaps, on the substrate. In either case, the response is only a few per cent of the total cytochrome *c* present. Cytochromes *a* and a_3 may show a reduction of a few per cent of the total amount but usually remain almost completely oxidized.

When all the ADP has been phosphorylated to ATP, state 4 ensues again with simultaneous changes in the steady-state oxidation-reduction levels and a fall of O_2 consumption. State 4 to state 3 transitions (followed by the reverse transitions) can be produced by ADP additions until the oxygen in the cuvette has been consumed, at which point all the components of the respiratory chain become fully reduced (state 5). The characteristic parameters of these states and the steady-state oxidation-reduction levels of cytochromes *a*, *c*, and *b* and of the flavoproteins and DPNH are compiled in Table I, which is adapted from the paper of Chance and Williams (1955).

TABLE I

METABOLIC STATES OF MITOCHONDRIA ISOLATED FROM GUINEA PIG AND RAT LIVER AND STEADY-STATE LEVELS OF SOME RESPIRATORY PIGMENTS

Adapted from Chance and Williams (1955), Tables I and II

	State 1	State 2	State 3	State 4	State 5
O_2 level	Aerobic	Aerobic	Aerobic	Aerobic	Anaerobic
ADP level	Low	High	High	Low	High
Substrate level	Low endogenous	Approaching 0	High	High	High
Respiration rate	Slow	Slow	Fast	Slow	0
Rate-limiting component	Phosphate acceptor	Substrate	Respiratory chain	Phosphate acceptor	Oxygen
			Per cent reduced		
Cytochrome *a*	Variable	~0	~5	~0	~100
Cytochrome *c*	Variable	~0	8	13	~100
Cytochrome *b*	Variable	~0	16	36	~100
Flavoprotein	Variable	~0	24	41	~100
DPNH	Variable	~0	54	~100	~100

In the present paper the reactions of mitochondria isolated from the leg muscles of the toad are first examined in an attempt to verify for these mitochondria the findings of Chance and Williams. Then it is attempted to extend these observations to the mitochondria in toad sartorii aged for about 2 days in the cold, which show a more simple response to contractile activity. Subsequently the more complicated situation in freshly excised muscles of the toad, frog, and turtle will be discussed. Finally some pertinent biochemical differences produced by the aging treatment will be examined.

METHODS

I. *Instrumentation*

The experiments described in this paper were performed with the "double beam" spectrophotometer developed by Dr. Britton Chance. In this instrument, mono-

chromatic beams of two different wave lengths are alternately passed through the sample to fall on one photomultiplier. The signals are subtracted and the difference recorded. In the work with the cytochromes, for example, one wave length is set at the absorption peak of one component of the chain, while the other (the reference beam) is set at an indifferent wave length, usually not more than 20 mμ away. The difference in the absorption of the two is recorded as a function of time. This instrumentation, therefore, furnishes information on the kinetics of the absorption

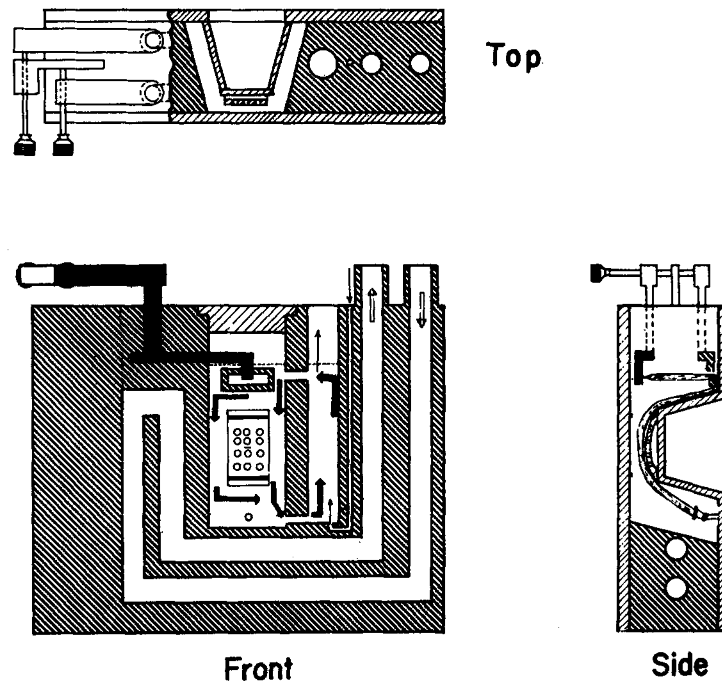

FIGURE 1. Muscle holder for kinetic measurements with the differential, double beam spectrophotometer. For description, see text. Materials in cross-section indicated as follows: solid, metal; heavily hatched, black acrylic plastic; lightly hatched, clear acrylic plastic (plexiglass). Heavy solid arrows show the stream pattern of Ringer's solution, line arrows, the gas flow, double line arrows the flow of the cooling water.

changes; *i.e.*, after contractile activity or upon the addition of reagents. For a more detailed description, see Chance (1951, 1954).

A muscle holder different from the one used for the split beam instrument is required for this type of instrumentation. It was specifically designed by Mr. V. Legallais for the reduction of the wave length non-specific optical changes and for the simultaneous recording of isometric tension. The first requirement is met by placing the photocell as close as is practicable to the muscle and by reducing the gross movements of the muscle as far as possible. Tension development is measured by means of strain gauges on a flexible beam.

The cell consists of two pieces (Fig. 1). One is a flat plate of lucite to which the

assembly for securing the muscle is attached. The other consists basically of a heavy piece of opaque lucite containing a cavity which fits around the muscle assembly. In addition, it has one channel for pumping the Ringer's solution and others for the flow of water from a constant temperature bath. The facing of this second piece is again a sheet of transparent lucite. The light beam passes through a cavity in the muscle assembly, through the muscle, across the facing plate in the second part, and from there, falls upon the window of the photomultiplier. The two parts of the cell are carefully machined, thus giving a good seal with minimum application of silicone or stopcock grease.

When the optical window of the end-on photomultiplier is brought close to the preparation, the solid angle subtended by the photomultiplier is increased. This is important for the reduction of both light scattering and light diffraction effects. In this cell the distance between the near surface of the muscle and the photomultiplier window is reduced to 6 mm. From any point in the sample the photomultiplier subtends an angle of 152°.

In order to reduce gross movements in the optical field and prevent thickening during contraction, the muscle can be stretched to about 110 per cent of rest length. That part through which the light beam passes lies over a curved, perforated sheet of lucite. If necessary, a piece of nylon netting can be pulled against the muscle to reduce movements of the fibers at the free surface. This last precaution, however, or that of stretching the muscle to >110 per cent rest length, was found to be superfluous in most cases.

One end of the muscle (the pelvic end of the sartorius) is tied to the lucite frame, while the other end is attached to a thin beam of lucite which bears strain gauges for the registration of isometric tension. The end of this beam bears a slotted bar through which an extra long muscle can be passed to be attached to another beam. The last two beams can be moved, with respect to each other and to the lucite frame, by means of lever arms attached to the outside of the cell. In this way, muscles of various lengths can be accommodated and the initial length can be adjusted conveniently during the course of an experiment.

Tension is measured by two strain gauges, one on each side of the beam. The gauges (Baldwin SR4-A-16) form two members of a Wheatstone bridge and are activated by 6.3 v 60 cycle AC. The signal caused by imbalance of the bridge is amplified by an AC amplifier–demodulator–DC amplifier system similar to that used for the recording of the spectroscopic changes. Although the limited bandwidth may introduce a slight distortion of the slope of the contractile phase of the fastest myograms (frog sartorius at temperatures >20°C), the gross features of even these are not obscured.

Stimulation is produced *via* two cathodes one at each end of the curved piece of lucite. The anodes lie on the other side of the muscle in the wall of the chamber. The stimulus passes, therefore, across the muscle. In the frog or toad sartorius excitation is thus produced simultaneously close to the pelvic origin and close to the point of entrance of the nerve. Stimulation is produced either by condenser discharges or square wave pulses.

The muscle is immersed in rapidly flowing Ringer's solution. The solution is moved by means of a gas lift or a small motor-driven propeller in a separate, vertical

channel outside the optical path. This channel is connected to the main chamber by two small horizontal channels, one at the top and one at the bottom. To insure adequate movement of the solution in back of the perforated lucite sheet, a small deflector is built into the mainstream. The performance of this flow system was checked by adding a drop of India ink to the solution. Using a moderate stream of bubbles or the motor-driven propeller, the entire solution appeared homogeneous in 5 to 10 seconds.

II. *Biological Materials*

Sartorius muscles of the frog and the tropical toad and the coraco-hyoideus muscles of red-eared and painted turtles were used for observations on intact muscles. For the experiments with isolated mitochondria, preparations (5000 $\times$ *g* fraction) from the hind leg muscles of the toad were again employed. (For the description of these preparations and further conditions see Jöbsis, 1963.)

III. *Chemical Determinations*[1]

Creatine and creatine phosphate were determined in paired muscles according to Ennor's method (1957). The muscles were equilibrated for 2 to 3 hours at 20° with the bone attached, after splitting the pubic symphysis with a single-edge razor blade. During this period, 100 per cent O_2 was bubbled through the Ringer's solution. For control experiments, the muscles were then frozen and quickly weighed on a cooled watch glass on a Mettler analytical balance after bone and tendon had been clipped away with cooled heavy scissors or bone clippers. After a little practice, the weighing could be performed in less than 10 seconds. There was no sign of thawing until muscles had been on the cooled watch glass for more than 1 minute.

As a control on the method, determinations on 6 pairs were carried out immediately after the equilibration period. The results are shown in Table II.

The variability of the total creatine content between animals was higher than expected for a metabolite of such central importance in the energy metabolism of the tissue. An explanation may be sought in the physical condition of the animals, which was often poor, even at the time of arrival. In the laboratory they were kept in moist moss at room temperature (23 $\pm$ 2°C in winter, 22–33°C in summer). As a rule, no food was provided; but when live insects were offered, the death rate, usually highest in the 1st week after receipt, was not lowered.

The glycolytic capacity was assayed by the determination of lactic acid by the method of Barker and Summerson (Barker, 1957). An initial series of paired, fresh toad sartorii was frozen after a 2 to 3 hour equilibration period at room temperature in Ringer's with 100 per cent O_2. They were then broken into 4 pieces and weighed as described before. Three of the fragments were allowed to thaw in vials in an N_2 atmosphere. After 10, 20, and 40 minutes, the entire contents of the vials were re-frozen and analyzed for lactic acid. The rate for the three intervals was found to fall off logarithmically. Initial rates were, therefore, determined by extrapolation to

[1] Many thanks are expressed herewith to Mr. George Glassbrenner for his skillful performance of the creatine and lactate determinations.

zero time on a semilogarithmic plot. The means (± average deviation) of these rates for left and right leg muscles were 197 ± 21 mM/kg/hr. and 215 ± 18 mM/kg/hr., respectively. Individual pairs showed a slightly smaller variation: an average of 7 per cent.

Because of the remote possibility that a real difference exists between left and right sides, the muscles for the experiments on the effect of aging were randomly chosen as control and experimental.

TABLE II

FREE CREATINE AND CREATINE PHOSPHATE LEVELS IN PAIRED TOAD SARTORII, 2 TO 4 HOURS AFTER EXCISION

Results are given in millimoles per kilogram

No.	Free Cr	Total Cr	CrP	FCr/CrP	ΔFCr	ΔTCr	ΔCrP
1a	6.2	30.1	23.9	0.26	0.6	0.2	0.4
b	6.8	30.3	23.5	0.29			
2a	5.2	18.1	12.9	0.40	1.0	1.1	0.1
b	4.2	17.0	12.8	0.33			
3a	8.0	31.8	23.8	0.34	0.5	0.6	1.1
b	7.5	32.4	24.9	0.30			
4a	7.6	27.4	19.8	0.38	0.8	3.8	4.6
b	8.4	23.6	15.2	0.55			
5a	9.1	33.1	24.0	0.38	0.3	2.2	1.9
b	8.8	30.9	22.1	0.40			
6a	9.4	34.5	25.1	0.37	1.4	1.4	0
b	8.0	33.1	25.1	0.32			
Average	7.4	28.5	21.1	0.36	0.77	1.55	1.35

RESULTS

I. *Experiments with Isolated Mitochondria*

An attempt was made to verify the responses of the cytochrome and DPNH with mitochondria isolated from the hind leg muscles of the toad. The quality of these preparations, however, was not wholly satisfactory (Jöbsis, 1963), and some reservations about the results should be kept in mind. At best, these experiments are only a partial verification of the results described for other mitochondria (Chance and Williams, 1955; Chance and Baltscheffsky, 1958).

The particles were prepared by grinding the tissue with sand in isotonic sucrose and subsequent differential centrifugation (Jöbsis, 1963). The fraction sedimenting between 500 and 5000 × *g* in 10 minute spins was used through-

out. Oxygen consumption was assayed with a stationary O_2 electrode in a rotating cup.

The relative rates of oxygen uptake in the presence of various substrates are as follows: succinate = 100, DPNH = 53, α-ketoglutarate = 51, glutamate = 45, α-glycerophosphate = 15, β-hydroxybutyrate = 8, endogenous = 8. The measurements refer to the rates in the presence of excess ADP and P_i. The high level of oxygen consumption with DPNH indicates the absence of a diffusion barrier for entrance into the mitochondria. This raises the suspicion, in analogy with results on liver mitochondria (Lehninger, 1954), that the limiting membrane must have been damaged and rendered rather porous. This is in agreement with the absence of a real DPNH peak in the reduced-oxidized difference spectrum (Jöbsis, 1963), which was interpreted as being caused by a leaking out of endogenous pyridine nucleotides during the preparative procedure.

Attempts to determine the phosphorylating efficiency (P:O ratio) were unsuccessful due to the lack of a return to a lower rate of respiration after the addition of ADP. Addition of this nucleotide to the mitochondria in a condition in which only a phosphate acceptor is lacking (state 4 of Chance and Williams, 1955) results in an increased level of respiration (state 3). With succinate as substrate the rate of O_2 consumption in state 3 is 3 to 4 times the rate in state 4. However, no return to a state 4 condition could be found. In other words, the enhanced rate of oxidation is not decreased at a time when the added ADP should have been phosphorylated. This finding may either be due to a high extrinsic ATPase level caused by contamination with myofibrillar fragments or to a high ATPase activity of the mitochondria themselves. In the latter case the mitochondria would be truly uncoupled. It is likely that both possibilities contribute.

With the present preparation, changes in the oxidation-reduction levels of the cytochromes were also observed upon the transition from state 4 to state 3. The experiments were performed by adding ADP (to a concentration of 0.2 mM) to the suspension after state 4 had been attained by the provision of an excess of succinate and P_i. The reaction to the substrate is limited to a reduction of cytochrome *b* and flavoprotein of about 31 and 38 per cent respectively. The effect of ADP addition consisted of an oxidation of cytochrome *b* and flavoprotein and a reduction of cytochrome *c*. The oxidation of *b* was observed in all preparations and averaged 16 per cent of the total *b* present. The response of the flavoproteins could be recorded in 5 out of 11 preparations. The extent varied from 3 to 13 per cent. A reduction of cytochrome *c* could be shown in 9 out of 13 preparations and maximally amounted to 4 per cent. These changes in the oxidation-reduction levels lasted until the moment of anoxia caused by the consumption of all the oxygen. This is in

agreement with the absence of a return of the respiratory rate from state 3 to state 4.

The rate of oxygen uptake in state 3 varies with the ADP concentration when P_i is present in excess (14 mM). A typical Michaelis-Menten relation is revealed with a K_M of 46 μM ADP (Fig. 2). Similar experiments with varying P_i levels in the presence of excess ADP (1 mM) showed a K_M for phosphate of 710 μM.

For the interpretation of experiments on whole muscle, a variety of compounds known to occur in muscle, as well as the uncoupling agent 2,4-dinitrophenol (DNP), were screened for their ability to accelerate the rate of oxygen uptake by muscle mitochondria. The ability of these substances to produce this state 4 to state 3 transition was assayed with the O_2 electrode in the presence of succinate and 14 mM phosphate. Carnosine, creatine, AMP, hypoxanthine, inosine, and Mg^{++} (all in the range of 10^{-4} to 10^{-3} M) had no

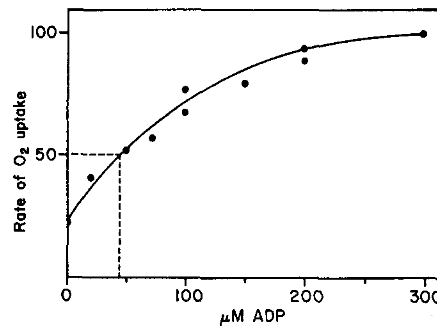

FIGURE 2. Rate of oxygen uptake by isolated toad skeletal muscle mitochondria as a function of the ADP concentration. The reaction medium contained 14 mM K-phosphate buffer, pH 7.2, 12 mM Na-succinate, 50 mM K Cl, 1 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid, and 50 mM sucrose. Temperature = 22°C.

significant effects on the respiratory rate when added to a mitochondrial suspension in state 4.

Addition of ATP (5×10^{-4} M) and DNP (10^{-4} M) produced an acceleration of the respiration of 290 and 240 per cent respectively. These values are similar to those obtained with ADP in this system. The high rate with ATP indicates a high ATPase activity in these preparations, a fact which was surmised before. DNP produces its effect by abolishing the need of a ~ P acceptor, probably by a hydrolysis of intermediate high energy compounds (Loomis and Lipmann, 1949; see also Slater and Hülsmann, 1959).

Calcium at a level of 0.1 mM produced a slight increase (approximately 50 per cent) in keeping with its known uncoupling action (Potter, 1947; Lehninger, 1949; Slater and Cleland, 1953). Higher concentrations produced an inhibition and precipitation of the mitochondria. This ion was included in this series of experiments because of its implicated role in muscle contraction (Heilbrunn and Wiercinski, 1947; Weber and Portzehl, 1954; Niedergerke, 1955; Bianchi and Shanes, 1959) and because of its effect on mitochondrial

metabolism. The increases of respiratory rate are small and negligible compared to the difference between state 4 and state 3 respiration caused by ADP addition.

II. *Respiratory Chain Responses in Aged Sartorii*

In recently excised toad muscles that have been allowed a rest period of 2 to 4 hours at room temperature (20–25°C) in well oxygenated Ringer's solution contractile activity was found to have discouragingly small effects upon the steady-state oxidation-reduction of the members of the respiratory chain. If lactate or pyruvate were added to the Ringer's (at 10 to 30 mM levels), greater changes in steady-state levels could be observed. After many months of work it was found that toad sartorii, aged for 36 to 48 hours at 0–3°C and supplied with substrate, were the most suitable for the observation of the kinetics of the respiratory chain components. The results under the latter conditions are, therefore, presented first.

For these experiments sartorius muscles of the toad were perfused, excised, and left to equilibrate for 2 to 4 hours at room temperature. The muscles were then kept at 0–3°C in Ringer's solution without substrates for the required time (usually 36 to 48 hours). During this period the muscles were kept at rest length or at a slightly extended length (approximately 110 per cent of rest length), and air was continuously bubbled through the Ringer's solution. After such treatment, the twitch tension at room temperature had fallen to about 90 per cent of the value which it had had after 2 to 4 hours' equilibration at 20–25°C. Otherwise, the myograms were identical with those of fresh muscles. In the absence of added lactate or pyruvate, the tension output of such aged muscles tended to deteriorate rather quickly in successive twitches. In the presence of added lactate or pyruvate, good twitch responses could be elicited for hours if periods of contractile activity were alternated with recovery periods. Tetanic contractions could be elicited several times if widely spaced in time, but, even then, tension had a tendency to drop with each successive tetanus.

Contractile activity of such aged preparations results in long lasting oxidations of cytochrome *b* and reduced diphosphopyridine nucleotide (DPNH), as measured at 430 to 410 and 340 to 374 mμ, respectively. Little, if any, effect can be recorded at wave lengths appropriate to the other members of the respiratory chain. An occasional exception was cytochrome *c*, which in some preparations may show a small change towards oxidation. A typical cycle of cytochrome *b* in response to 20 twitches is shown in Fig. 3 (top curve). This response was recorded from a toad sartorius aged for 42 hours at 2°C ($\pm$2°). The trace returned to the base line in about 75 minutes; further cycles could be elicited by a new series of twitches. In many preparations, however, this return is greatly delayed, or a new base line at a slightly more

oxidized level is established. The response of DPNH is similar to that of cytochrome *b* under these circumstances except that the early diphasicity is lacking; *i.e.*, the curve reaches a maximum directly and declines monotonically (see Fig. 5).

The response of the resting muscle to the addition of lactate or pyruvate is a reduction of cytochrome *b* and of DPN, while occasionally cytochrome *c* and the flavoproteins react in a similar manner but to a much smaller extent. The half-time for these reductions is about 15 to 25 minutes, depending partially on the thickness of the preparation. Quite frequently the trace does not become stable for 1½ to 2 hours. A similar response was noticed to a limited degree for succinate, but no effect could be recorded for other intermediates of the citric acid cycle or the glycolytic chain, nor for glutamate, α-glycerophosphate, β-hydroxybutyrate, or glucose.

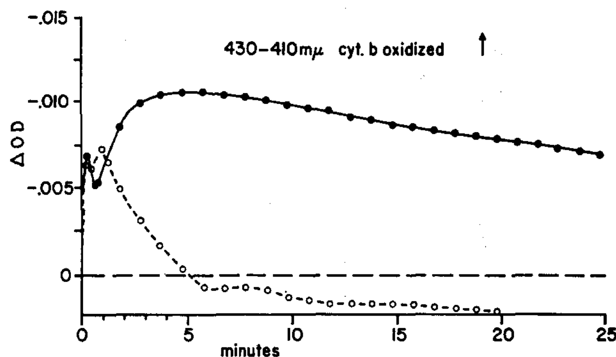

FIGURE 3. Responses of cytochrome *b* to 20 twitches in 20 seconds; aged toad sartorius, 20°C. Solid circles in the absence of added substrate, open circles after equilibration with 5 mM Na-pyruvate for half an hour.

The extent of this effect shows considerable variation from one muscle to the next. The limits were found to be from 3.5 to 32 per cent of the cyanide-reducible cytochrome *b* and from 2.4 to 17.8 per cent of the DPN concentration assessed similarly. In Fig. 4 the top curve shows the dependence of cytochrome *b* reduction in the resting state on the substrate concentration.

After the addition of pyruvate or lactate, the oxidation of cytochrome *b* and of DPNH in response to contractile activity is of approximately the same extent that it was prior to the addition, but it is of shorter duration. The lower curve of Fig. 3 shows such a cycle of cytochrome *b* half an hour after the addition of 5 mM pyruvate to the muscle from which the top curve had been first recorded. Afterwards the new base line is slightly more reduced than the level before the contractions. This feature usually disappears after several series of contractions and is probably caused by a slight increase in substrate level in the cell. The dependence on substrate concentration of some character-

istic quantities of such response curves is shown in the other curves of Fig. 4. These parameters are: $P_{supermax}$, the amount of oxidation of cytochrome *b* at the first maximum; P_{max}, the size of the second maximum; and $t_{1/2\ off}$, the time taken for the oxidation level to decrease to half the value of P_{max}.

It is clear from Fig. 4 that the amount of reduction of cytochrome *b* in the resting state depends on the substrate concentration up to approximately 8 mM of pyruvate. A similar effect can also be plotted for lactate and succinate, for which the curves usually flatten off around 5 and 25 mM, respectively. The differences in concentration needed for the saturation level may well depend on the rate of penetration of these substrates into the muscle fibers. This rate probably plays a role also in the time for half-maximal reduction of cyto-

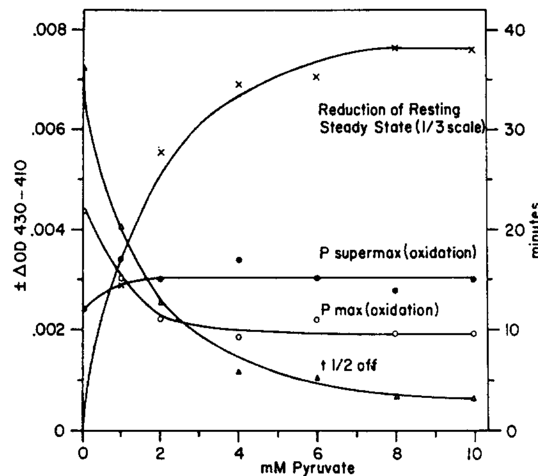

FIGURE 4. Parameters of cytochrome *b* as a function of external substrate concentration; aged toad sartorius, 23°C. $_{supermax}$ and P_{max} (both left ordinate) and $t_{1/2\ off}$ (right ordinate) refer to responses to 30 twitches in 15 seconds. The top curve shows increases in the reduction of cytochrome *b* under resting conditions (scaled down by a factor of 3).

chrome *b* after addition of substrate; *i.e.*, approximately 15, 12, and 35 minutes for lactate, pyruvate, and succinate, respectively.

Of quite considerable interest is the variation of $t_{1/2\ off}$ with the substrate concentration. Here again there is a saturation effect with a stable value being established at about 8 mM of pyruvate (Fig. 4). It appears that at this external level the intracellular concentration ceases to be limiting.

Similar effects of the addition of substrates both on the resting oxidation-reduction steady state and after contractile activity can be recorded for the pyridine nucleotides, while sometimes cytochrome *c* also becomes reduced to a very small extent.

From these findings the conclusion is drawn that in the aged muscles the substrate supply is limited. Addition of pyruvate, lactate, or succinate to the

Ringer's solution apparently overcomes this deficiency. The lack of similar effects for many other substrates which can be metabolized by isolated mitochondria (see below) suggests the existence of a diffusion barrier for these substances. Penetration through the cell membrane either does not occur or occurs at rates so low that the metabolic needs cannot be met. The relatively slow establishment of a steady level of cytochrome *b* reduction after the addition of succinate may well be due to a lower rate of penetration of this substrate.

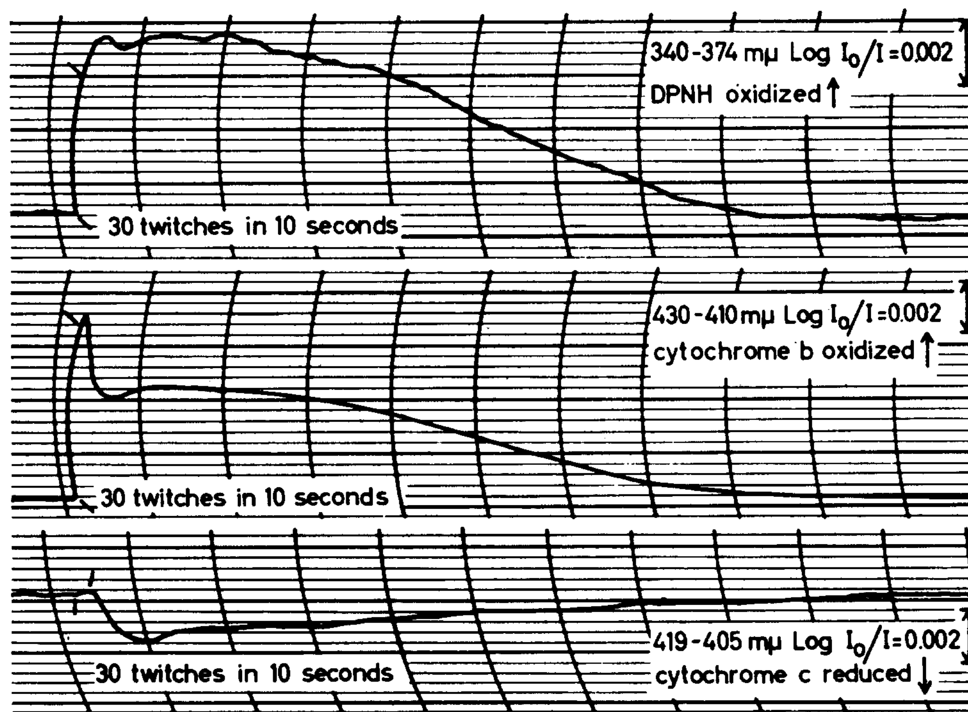

FIGURE 5. Responses of three members of the respiratory chain to contractile activity; aged toad sartorius, 5 mM pyruvate, 23°C. Time scale, 1 minute between curved lines of chart grid. The short diagonal lines indicate the times of the first and the last contraction.

In Fig. 5 the cyclic changes in response to 30 twitches are shown as observed at the appropriate wave length pairs for the pyridine nucleotides and for cytochromes *b* and *c*. The traces were recorded from one aged toad sartorius supplied with 5 mM pyruvate. It is interesting to note that all three responses have approximately the same duration. The shape of the cytochrome *b* curve is quite different from that of the other two because of the occurrence of the early maximal amount of oxidation ("supermaximum") followed by the longer lasting plateau.

Cytochrome *c*, in contrast to cytochrome *b* and DPN, exhibits a cyclic change towards a more reduced steady state in response to contractile activity. No reaction could be recorded consistently for the other components of the respiratory chain, although often a slight, inconclusive increase of absorption at 465 mμ (as compared with 510 mμ) was found.

CONTROLS ON THE OPTICAL DENSITY CHANGES

The above measurements were all made by registering the optical density changes at two wave lengths appropriate to each component. To ascertain more specifically the nature of the absorbing materials responding to the contractions, two types of checks were made: one showing the location and shape of the absorption peaks, the other establishing their identity by recording under conditions known to prevent changes in the steady states of the components of the respiratory chain.

For the first type of control, cycles were first recorded at wave lengths appropriate to the α peaks of cytochromes *b* and *c*; *i.e.*, at 564 to 575 and 550 to 540 mμ respectively. These were then compared with the responses of the same muscle at 430 to 410 and 419 to 405 mμ (the γ peaks). The data were found to check qualitatively: the measurements of the α peaks showed an oxidation of cytochrome *b* and a reduction of *c*. Quantitatively, the results were also satisfactory. From measurement of the records we obtained γ to α peak ratios of 2.9 and 7.3 for cytochromes *b* and *c* respectively. This agrees reasonably with ratios of 2.8 and 6.8 for these two components in the difference spectra. The configurations, as well as the durations, of the cycles were also found to be quite similar for the two components.

Further verification of the components responsible for these optical density changes comes from experiments in which the spectra are recorded over the region in question. In this case, the whole Soret region (400 to 450 mμ) and the 535 to 580 mμ part of the visible range were investigated, as well as the near ultraviolet around 340 mμ. Such experiments were performed by recording a series of cycles at a number of overlapping wave length pairs (400 to 405, 405 to 410, 410 to 413 mμ, etc.) or by keeping one wave length constant while varying the other (425 to 400, 425 to 405, 425 to 430, etc.). The size of the deflections at various times after the series of twitches can then be plotted against the wave lengths. The results of such experiments are shown in Fig. 6.[2] Because of the considerable amount of activity involved, the visible and Soret regions presented in Fig. 6 were obtained from two different muscles.

[2] In the direct records the optical density changes are reported in the negative sense in agreement with laboratory custom. This practice has its origin in the fact that a decrease in optical density at the measuring wave length (*i.e.*, 430 mμ for cytochrome *b*) gives rise to an increase in photomultiplier current. In plotting the spectra, however, it was thought more advisable to show a reduction of a cytochrome as an absorption peak in adherence to standard practice.

The onset of fatigue precluded the completion of both sets on a single preparation.

It is seen that at 30 seconds after the start of the activity, the time at which the supermaximum at 430 to 410 or 564 to 575 mμ has reached its greatest extent, the only features of the spectra are troughs at 431 and 564 mμ, which are attributed to an oxidation of cytochrome *b*. In the second curve, depicting the optical density changes 50 seconds later, the oxidation of *b* has been

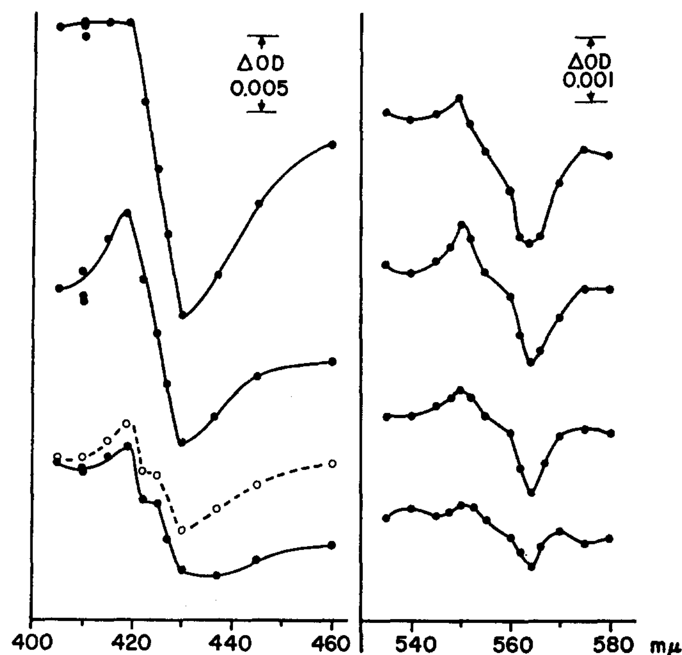

FIGURE 6. Spectral characteristics of the recovery cycles at selected wave lengths in response to 20 twitches in 20 seconds; aged toad sartorii, 5 mM pyruvate, 20°C. From the top on down the curves represent the spectra at 30 seconds, 80 seconds, and 4 minutes (approximately $t_{1/2\ \mathrm{off}}$) after the first twitch. The broken line in the set on the left is the bottom curve after the application of a base line correction. In the visible region the relatively greater stability of the base line allowed a meaningful representation of the situation at 7 minutes (bottom curve).

decreased slightly, but peaks identified with a reduction of cytochrome *c* have developed at 419 and 550 mμ. Later these two components return again to the resting steady-state levels. A slight shift of the base line is usually found, which becomes more noticeable when the peaks of cytochromes *b* and *c* have subsided again. This optical effect, which has a different time course than that attributed to members of the respiratory chain, is especially prominent in the Soret region and might be ascribed to physical effects such as scattering and refraction. Subtraction of the effect, as shown in the left, bottom record

of Fig. 5, clarifies the position of the cytochrome *b* and *c* peaks at a time when their intensity is lower. The extent of this base line shift is extremely variable between preparations and may either increase or decrease with time in one muscle. Its cause is not understood.

No other absorption peak develops in the two regions. From these spectra we can see that cytochrome a_3 (445 mμ) does not respond noticeably. (Nor does cytochrome *a* (605 to 630 mμ) undergo changes of its steady-state oxidation-reduction level, as mentioned before.) The flavoproteins (measured at 465 to 510 mμ) usually do not show a clear cut response. The tilting of the base line will produce a noticeable effect at this wave length pair, although with different time characteristics. Nevertheless, the occurrence of a slight oxidation of the flavoproteins was occasionally suspected, especially in muscles exhibiting a large cytochrome *b* effect. The absence of a clear cut response is, perhaps, not too unexpected considering the low flavoprotein concentration of these muscles and the small extinction coefficient.

Similar exploration around 340 mμ showed a trough with a minimum at 335 to 340 mμ much resembling that caused by an oxidation of DPNH.

In the second type of control experiment, electron transfer *via* the respiratory chain can either be blocked with cyanide or a lack of O_2, or made maximal by the addition of 2,4-dinitrophenol (DNP). Under anoxic conditions or in the presence of cyanide (7.5 mM), the cyclic responses at 419 to 405, 430 to 410, 550 to 540, and 564 to 575 mμ were completely abolished. A slight, continuous change towards increasing absorption at 430 and 564 mμ was usually recorded during anoxia. This effect continued long after the other members of the cytochrome chain were reduced and was not accelerated by activity. It is attributed to the slow reduction of the unknown compound(s) absorbing at 560 to 562 and 432 to 436 mμ, which persists long after the completion of the reduction of other members of the respiratory system (Jöbsis, 1963). Under these conditions (CN^- or N_2), the events at 340 to 374 mμ, where the reduction of the pyridine nucleotides is monitored, are similar to those at 419 to 405 mμ; *i.e.*, an absence of responses to series of twitches and a stable base line after the attainment of the anoxic state. It should be pointed out that, after the first few contractions under anoxic conditions or in the presence of cyanide, the twitch tension developed by the aged toad sartorii decreases rapidly. Therefore, only a few checks can be made with cyanide, and anoxic experiments can only be performed if aerobic recovery intervals are allowed regularly. Addition of glucose does not improve the anoxic performance.

Upon the addition of DNP (5×10^{-4} M) to the resting muscle, oxidations of cytochrome *b* and of DPNH and a reduction of cytochrome *c* can be recorded. The use of this uncoupler of oxidative phosphorylation is en-

cumbered by a rapidly starting deterioration of the preparation. Even without contractile activity (except for occasional testing with a single twitch), the twitch tension starts to decline soon and will approach zero after as few as 30 or 40 twitches. In order to perform meaningful experiments, the temperature of the preparation had to be lowered (to about 5°C) during the 20 to 30 minutes required for the penetration of the DNP. The effect of the uncoupler was then tested immediately after the temperature had been returned to about 15°C. DNP proved to abolish the cycles of both cytochromes *b* and *c* and of DPNH in response to contractile activity. This is in agreement with the finding in isolated mitochondria and also in intact tissues that this agent produces maximal respiration. In a maximally respiring system, further increases by means of contractile activity with concomitant changes in the oxidation-reduction levels of the members of the respiratory chain would not be expected.

From the results of these various control experiments it must be concluded that the optical events monitored at 430 to 410, 419 to 405, and 340 to 374 mμ do depict the responses of cytochromes *b* and *c* and of DPNH, respectively. These members of the respiratory chain respond to contractile activity with a cyclic change in their oxidation-reduction level.

QUANTITATIVE CORRELATIONS

The responses of cytochrome *c* and DPNH are qualitatively independent of the amount of activity, *i.e.* the form of the cycles remains the same, only the size and duration of the responses vary. The cytochrome *b* cycles, however, change qualitatively with the number of twitches (Fig. 7). After one or a few twitches, the curve declines monotonically from a rapidly reached maximum. The response to a considerable number of twitches consists of two phases, as was previously shown in Figs. 3 and 5. An initial, short lasting maximum (supermaximum) is followed by a more extended, secondary maximum which sometimes has the appearance of a plateau. The cause of the "supermaximum-maximum" type of response exhibited by cytochrome *b* is not understood. This type of response of cytochrome *b* has been observed by Chance and Hollunger (1963) upon the addition of ADP to guinea pig liver mitochondria in the presence of substrate and P_i, but not for preparations derived from other sources. This may indicate a variation of the kinetic parameters in different mitochondria. However, within the muscle cell a buffering effect of creatine phosphate in equilibrium with the more labile high energy phosphate stores could very easily be the underlying cause.

Nevertheless, it is clear that for all three components (cytochromes *b* and *c* and DPN) the extent and duration of the cycles vary directly and consistently with the number of twitches: more intense activity results in a more intense

and longer lasting reaction of the respiratory chain during recovery. This holds true also for cytochrome *b* even though the cycles vary in shape with varying amounts of activity.

Calculation of the amount of substance stimulating the respiration (*i.e.*, ADP, P_i, or other) produced per twitch can best be made from the number of twitches which give half-maximal effect. For this purpose the dependence of the intensity of the responses of cytochromes *b* and *c* and of DPNH on the number of contractions was ascertained in the presence of excess substrate (Fig. 8). This amounts to determining the K_M of the reaction. Such experi-

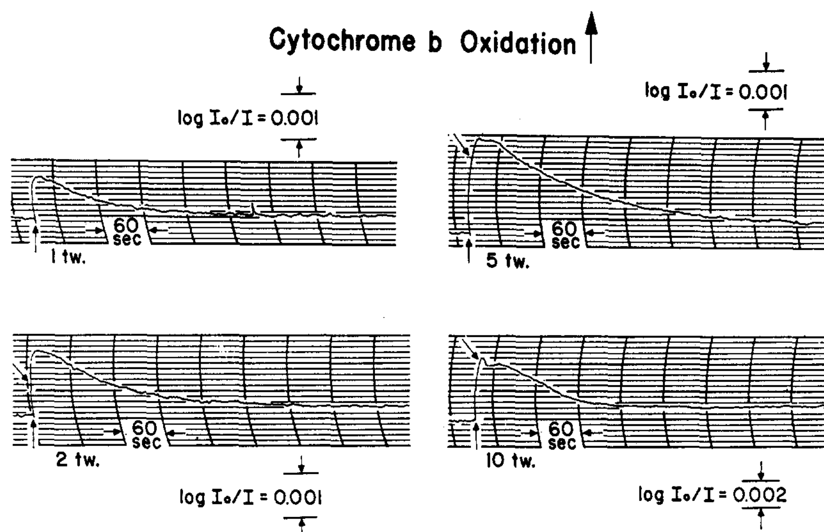

FIGURE 7. Responses of cytochrome *b* to increasing numbers of twitches; aged toad sartorius, 20°C, 10 mM pyruvate. Stimulation was at a rate of 1 per second. Note the changes in sensitivity in the last record.

ments have shown that this value is reached in 3 to 6 twitches (the average about 5).

Summing up the observations on aged toad sartorii, we can state that these preparations appear to lack the ability to transfer reducing equivalents at an adequate rate from the endogenous substrate to the respiratory chain. The ineffectiveness of glucose as an exogenous substrate, as well as the inability of these muscles to maintain good tension in the presence of glucose under anoxic conditions, tends to imply a dysfunction of the glycolytic system. (For further evidence, see below.) In the presence of lactate or pyruvate, cytochromes *b* and *c* and the pyridine nucleotides respond to contractile activity with long lasting cyclic changes in their steady-state oxidation-reduction levels. Under these circumstances cytochrome *b* and DPNH become temporarily more oxidized, while cytochrome *c* becomes more reduced. In other

words, a cross-over point (see Chance and Williams, 1955) has been demonstrated to exist between *b* and *c*. Furthermore, the unknown, reducible compounds with peaks at 560 to 562 and 432 to 436 mμ do not respond to contractile activity with changes in degree of reduction.

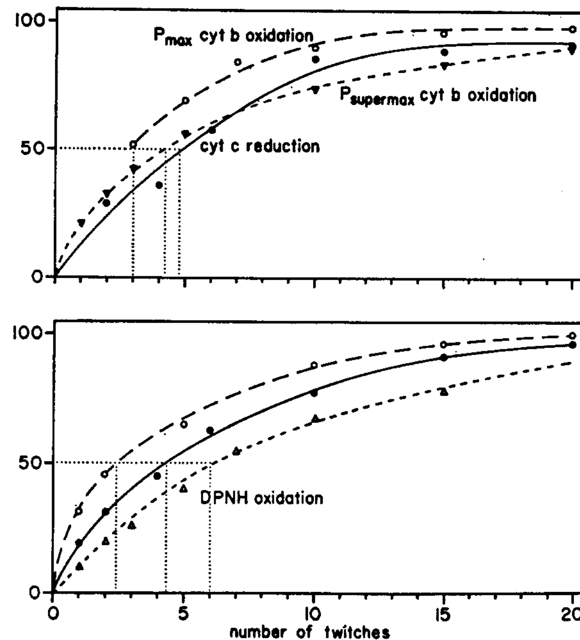

FIGURE 8. Representative saturation curves of the responses of cytochromes *b* and *c* and of DPNH to contractile activity at 1 twitch per second. Aged toad sartorii, 15 mM pyruvate, 18°C. The response to 30 twitches is maximal and is given the value of 100 per cent. Dotted lines indicate K_M values. DPNH measurements for 3 different muscles; cytochrome *b* curves were measured on the muscle which gave the middle curve for DPNH; the cytochrome *c* responses shown were derived from a fourth preparation.

III. *Respiratory Chain Responses in Recently Excised Muscles*

In these experiments the muscles were allowed an equilibration period of 2 to 4 hours in Ringer's solution with 100 per cent O_2 at room temperature before the experiment was started. This period of rest improves reproducibility.

The most certain single generalization which can be made about the results with fresh muscles is that there exists a large degree of variability in the quantitative aspects of the response of the respiratory chain to contractile activity. This variability occurs mostly between preparations; the data are usually internally consistent and reproducible for each experiment. The main exception to this rule is the level of oxidation of cytochrome *b* in resting muscle. Frequently, this level may show slow changes to either the reduced

or the oxidized side, and after a cycle induced by activity the trace often does not return to the base line. Cytochrome *c* and DPNH exhibit a greater stability.

TOAD SARTORIUS

In the fresh toad sartorius the effect of twitches is again limited to changes in cytochromes *b* and *c* and in the pyridine nucleotides. Of these, the response of cytochrome *c* is most straightforward: activity always results in a reduction. The amount of reduction may vary between preparations to the point that the effect may be hardly discernible, but the response always remains a reduction. The form consists of a relatively rapidly attained maximum followed by a monotonic return to the base line (Fig. 9). The response of the

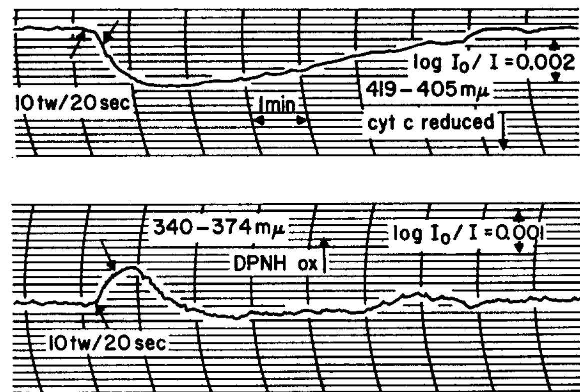

FIGURE 9. Responses of cytochrome *c* and DPNH of a fresh toad sartorius; 20°C, no substrate added.

pyridine nucleotides is more variable; most often it consists of a small short lasting oxidation during and immediately after the activity, followed by a later reduction phase which often gives way again to an oxidation before the stable base line level is regained (Fig. 9). The extent of these changes in the oxidation-reduction level of DPN is quite small, usually not more than a per cent of the total DPN reducible with cyanide. Occasionally a preparation will show a monophasic oxidation quite comparable to those seen in aged toad sartorii supplied with substrate, although sometimes a slight reduction may occur before the trace returns to the base line level. With moderately increased amounts of activity the first type of response (bi- or triphasic) shows no qualitative and but little quantitative change. A slight increase in amount of reduction is, perhaps, the most consistent effect of larger numbers of twitches. Muscles exhibiting the second (monophasic) type of DPNH

response show a consistent increase of oxidation with increasing number of twitches.

The behavior of cytochrome *b* is more complicated and only partially reminiscent of the responses in aged muscles. One twitch produces a long lasting oxidation which declines slowly in the course of about 6 minutes (Fig. 10). With larger amounts of activity the response begins to show a more complex character, similar to that of responses in aged muscles. Two distinct phases appear, starting in the record of the response to 5 twitches. This record and the one of 10 twitches show distinctly a rapidly attained supermaximum,

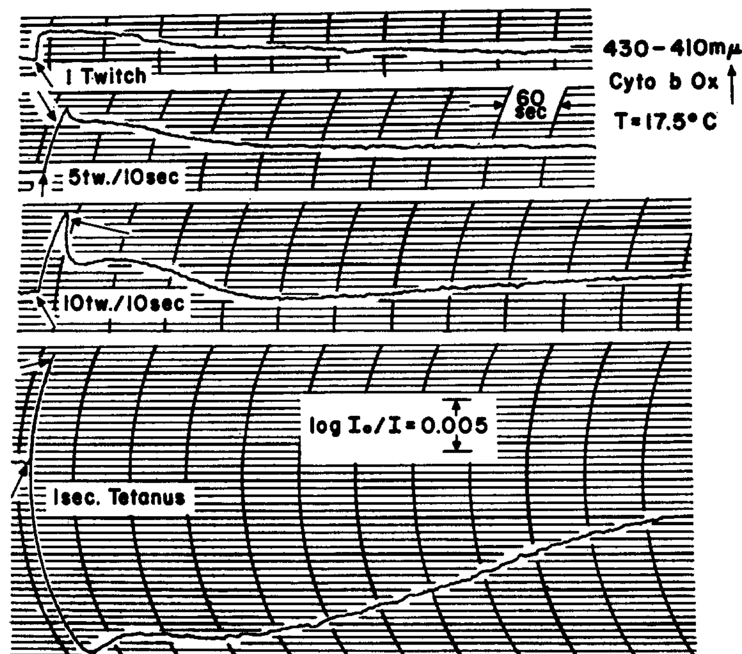

FIGURE 10. Responses of cytochrome *b* of a fresh toad sartorius; no substrate added. For the tetanus the frequency of stimulation was 40 per second.

followed by a plateau or second maximum. In the latter record it is seen that the trace goes below the base line (*i.e.*, a reduction) before it returns to approximately the base line level. With larger amounts of activity this tendency towards a reduction becomes greater. During a tetanus of 1 second duration, the trace first shows an oxidation followed by a precipitous fall to a greatly reduced level from which it returns only slowly. After about 40 minutes the level has returned again to approximately the steady-state level which cytochrome *b* had before this activity. Nevertheless, an indication of the secondary maximum of oxidation appears to be superimposed on the reduction at approximately 1 minute after the activity. It should be empha-

sized that this sharp reduction is not caused by partial anoxia due to insufficient oxygen tension in the inner regions of the muscle. This point can easily be checked by monitoring cytochromes *a* or a_3 during such a cycle. These two components showed no reduction under these circumstances.

The records of Fig. 10 also illustrate the instability of the base line when recording the oxidation-reduction level of cytochrome *b*. It can be seen in the last 3 traces that the base line is not regained exactly. After 5 and 10 twitches the level remains slightly more oxidized, while after the tetanus the distinctly more reduced conditions remained for at least 40 minutes.

The variability between individual toad sartorii is especially noticeable in the behavior of cytochrome *b*. The response to one twitch, when detectable, is almost always an oxidation, but its extent may be quite different from one preparation to the next. Also, the onset of the reduction response to increased numbers of twitches varies considerably. For 5 twitches a reduction is usually not yet observed in any part of the cycle. In some preparations this is still the case after 20 or even 30 twitches, while in others the response after as few as 10 twitches is largely a reduction, except for the initial supermaximum. (Out of close to 200 toad sartorii examined, 2 specimens responded with a typical biphasic response of cytochrome *b* wholly in the direction of a greater reduction. In both these cases cytochrome *c* showed a typical reduction cycle, while for DPNH the usual picture of a slight oxidation followed by a small reduction was recorded. In these cases the cross-over point apparently was shifted from between *b* and *c* to a position before cytochrome *b*.)

The inconstancy of the base line, as well as the differences between preparations, can be ameliorated by the addition of lactate or pyruvate. In the completely resting muscle, the effect of these substrates is commonly limited to a reduction of DPN and cytochrome *b*. The extent of this reduction is variable and runs from 4 to 9 per cent of the cytochrome *b* and from 0 to 22 per cent of the pyridine nucleotides reducible by cyanide. Cytochrome *c* is usually unaffected, though at times a small reduction (< 2 per cent) can be observed.

After the addition of substrate the oxidation of cytochrome *b* in response to contractile activity is increased, and the number of twitches at which the first reduction appears becomes larger. The DPNH response is usually less affected, but a slightly increased intensity of the oxidative part of the cycle appears to be the rule.

Control experiments, as described above for the aged muscles, bear out the fact that the responses are truly of the three components to which they are assigned. A phenomenon differing from that seen in the aged sartorii is observed for DPN during complete anoxia. Upon the replacement of O_2 by N_2, DPN is reduced. After a rapid, initial phase the trace will continue to show a slowly continuing increase in DPNH formation at a time when cytochromes *c*, *a*, and a_3 have already reached a stable level. If contractile activity

is now induced, a quick cycle of reduction and reoxidation followed by a slower, lasting reduction is seen. These observations indicate that there are apparently two fractions of pyridine nucleotides in the tissue (see also Jöbsis, 1963). A likely assumption is that these are the intra- and extramitochondrial DPN. The cycles observed in anoxia are interpreted as the activity of the extramitochondrial (cytoplasmic) DPN during glycolysis.

An obvious control experiment, which would provide confirmation or refutation of many of the above conclusions, would be the inhibition of glycolytic activity by iodoacetate (IAA). It proved, however, that the addition of this agent produces such extensive, continuous changes in the general optical properties of the tissue that meaningful observations are precluded, especially at the wave lengths appropriate to DPNH. One of the better records of the effect of IAA on the cytochrome *b* response is shown in Fig. 11.

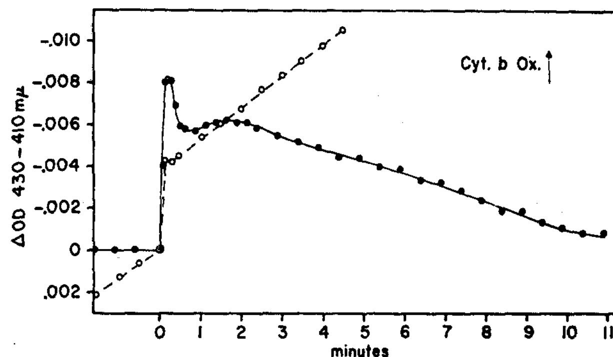

FIGURE 11. Effect of iodoacetate on the response of cytochrome *b* to 10 twitches in 10 seconds; fresh toad sartorius, 14°C, no substrates added. Solid circles, before; open circles, after 40 minutes' exposure to 5.0×10^{-4} M IAA.

That the drift of the base line was not caused by a continuous oxidation of cytochrome *b* was shown by similar recordings at a variety of wave lengths. The sudden effect associated with the contractions appears to be a lasting oxidation of this component. The failure of a true cyclic response of oxidation followed by a return to the base line may be interpreted as an indication of lack of substrate caused by the inhibition of glycolysis. Under similar conditions the response of cytochrome *c* was abolished as far as could be ascertained.

TOAD SARTORIUS: QUANTITATIVE CORRELATIONS

In normal, fresh toad sartorii a long tetanus produces a long lasting reduction of cytochrome *b* and frequently also of cytochrome *c* and of DPNH. Anoxia did not occur, as was evident from control experiments on cytochrome *a*. The return to the resting level is extremely protracted and variable. In some

preparations a persistent, more reduced level may be attained, especially for cytochrome *b*. In most, however, the trace will gradually return to the base line in a matter of 1.5 to 3 hours in the absence of added substrates. Moderate amounts of activity actually speed up rather than impede this return. In the presence of added substrate (lactate or pyruvate), when the oxidation-reduction levels are already more reduced, such effects of tetanic activity are still present. They are smaller, however, and the return is accelerated to something less than an hour.

The weight of the above experiments tends to show that the substrate level in the cell is labile and is increased by contractile activity. Under these

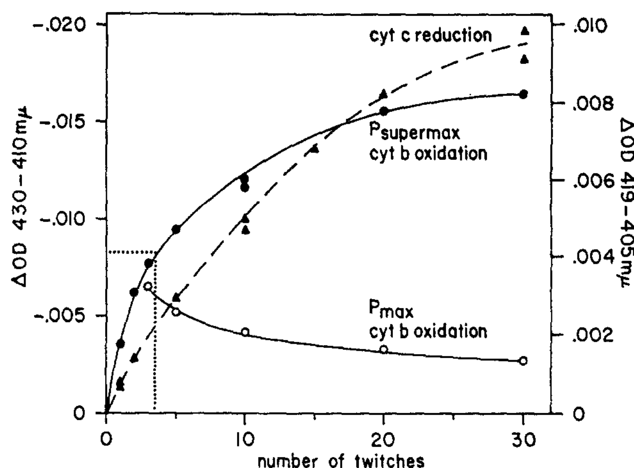

FIGURE 12. Saturation curves of the responses of cytochromes *b* and *c* to varying numbers of twitches in a 20 second period; fresh toad sartorius, 20°C, Na-pyruvate, Na-lactate, and Na-succinate at 15 mM each, osmotic pressure of Ringer's compensated for by lowering the NaCl concentration. Left ordinate, cytochrome *b*; right ordinate, cytochrome *c* measurements. Dotted line indicates approximate K_M value.

circumstances it is difficult to assay the amount of activity which gives half-maximal effect on the respiratory chain, as was done for the aged muscles (see Fig. 8). It turned out that the supermaximum response of cytochrome *b* was a reliable indicator only in the presence of greatly excessive concentrations of substrate (Fig. 12). The oxidation of DPNH and the plateau ("P_{max}") of the oxidation of cytochrome *b* are counteracted by the reducing influence of the augmented substrate levels. The response of cytochrome *c* is complicated by the additive features of the two responses, both being in the direction of greater reduction. The supermaximum value of cytochrome *b* is apparently reached rapidly enough to be complete before the influence of the increased substrate level is felt. The stimulation was delivered, therefore, during a constant period (20 seconds) rather than at a constant frequency. The re-

sulting saturation curves reach a half-maximal level between 3 and 5 twitches, 4 on the average.

FROG SARTORIUS

In frog sartorii the responses are generally smaller than in fresh toad sartorii, while, furthermore, the aging treatment at low temperatures does not produce an enhancement of the responses. At room temperature the cycles are usually too small to be recorded with certainty, for which the lower concentrations of DPNH and the cytochromes in the frog muscles (Jöbsis, 1963) are probably partially responsible. The addition of lactate or pyruvate to resting muscle resulted in a cytochrome *b* reduction of 1 to 8 per cent. Similar observations have been made by Weber (1957) and by Weber and Chance (1963). Subsequent activity sometimes resulted in an oxidation of cytochrome

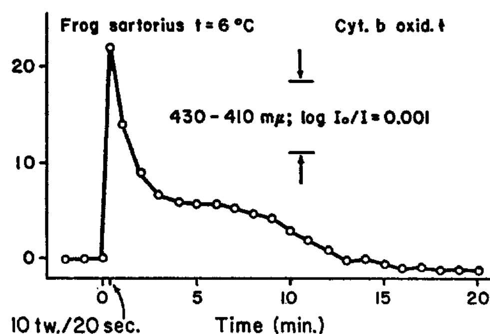

FIGURE 13. Frog sartorius; response of cytochrome *b* to 10 twitches in 20 seconds; 10 mM Na-pyruvate.

b and a reduction of cytochrome *c*, but usually the cycles, if any, were of approximately the same magnitude as the noise level.

The situation changes when the temperature is lowered to 4-10°C. Here we regularly observe a definite oxidation of cytochrome *b* (Fig. 13). The typical response is much like those described for the toad sartorii. There is a rapid phase of oxidation followed by a longer lasting plateau of oxidation above the resting steady state. The cycles of *c* are either too small to be recorded or are absent. An oxidation of DPNH can commonly be observed at these temperatures, as was first shown by Chance and Connelly (1957). At yet lower temperatures (0°C) the responses may become slightly more extensive but greatly protracted, which makes them difficult to distinguish from the fluctuations of the base line.

TURTLE CORACO-HYOIDEUS

The responses of the respiratory chain of the turtle coraco-hyoideus are usually small but similar to those of the amphibian sartorii described above. The

results are depicted in Fig. 14. Because of the less parallel arrangement of the fibers and a considerable myoglobin content, the motion artifact of the contractions is often larger than in the sartorii and consists of a rapid deflection and a slower return to the base line. Although this adjunct complicates the observation of the changes in steady state of the members of the respiratory chain, it does not prevent it. The general form of the responses of cytochromes *b* and *c* is quite similar to those already described for the other muscles. Exclusive of the motion artifact, there is a complex, oxidative response of cytochrome *b*: an early maximum followed by a longer plateau. Cytochrome *c* shows again a reduction. In this muscle it was also possible to record responses of the flavoproteins, as shown in the figure. Although its extent is small, an

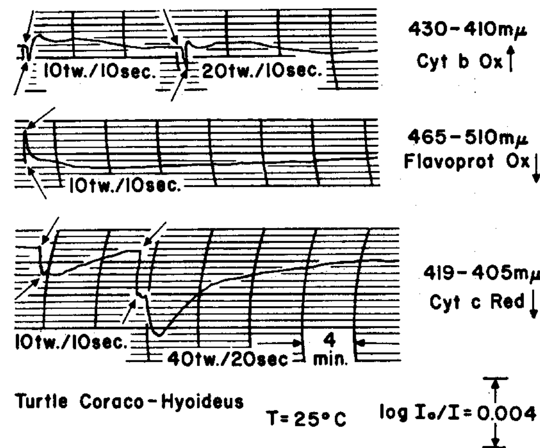

FIGURE 14. Turtle coraco-hyoideus; responses of cytochromes *b* and *c* and of flavoprotein in response to contractions. N.B. Recorder speed was 4 times slower than in other recordings depicted in this paper.

oxidation can be distinguished after the motion artifact has subsided sufficiently. The flavoprotein cycle has approximately the same duration as that of cytochrome *b*.

The response of cytochrome *c* is relatively more prominent than that observed in other muscles, while the cytochrome *b* cycles are smaller. Ten twitches produced a reduction of 8.5 per cent of the total cytochrome *c* concentration. The response to 40 twitches, shown in Fig. 14, is an example of the form of the curve occurring during hypoxia: a sudden, rapid reduction followed by a relatively slower reoxidation. The time course of the anoxic period was checked at the wave lengths for cytochrome *a*.[3]

[3] These experiments were performed in the muscle holder described by Chance and Connelly (1957). In this holder the muscle is slightly compressed between two perforated "lucite" plates. This arrangement cuts down the surface available for O_2 diffusion, which may account for the partial anoxia.

IV. *Creatine Phosphate and Glycolysis in Aged and Fresh Toad Sartorii*

Creatine phosphate and creatine concentrations and the rate of lactate formation were measured between paired toad sartorii, one fresh and one aged. These two parameters were chosen because of their outstanding importance in the energy metabolism of the tissue. Figures on the CrP content, and, especially, the ratio of creatine to CrP are considered to be a good indication of the over-all state of the $\sim$ P level of the tissue. It appeared possible that the much greater responses of the aged muscles might be related to a markedly lower CrP concentration. The "buffering" action of this $\sim$ P store on possible increases in the ADP content after contractions would then be reduced considerably, and higher levels of ADP would be accompanied by greater changes in the oxidation-reduction levels of the cytochromes. The capacity for lactate formation was chosen as an indication of the condition of the glycolytic system.

CREATINE PHOSPHATE

For the CrP determinations paired toad sartorii were equilibrated for 2 to 3 hours at 20°C under constant bubbling of 100 per cent O_2. One muscle was tied to a glass rod at 100 to 110 per cent body length and stored at 0–3°C. Air was bubbled through, and the solution was changed at least once. The other muscle was mounted in the holder; 20 isometric twitches were induced, and the response of cytochrome *b* was recorded. Pyruvate to a 10 mM level was then added to the Ringer's solution; the reduction of cytochrome *b* was recorded, and after time for equilibration (usually 40 to 60 minutes), the responses of *b* upon 10 to 20 contractions were recorded. The total amount of the quickly reduced cytochrome *b* was measured from the optical density changes caused by N_2-anoxia speeded up by 10 to 20 contractions. Subsequently 100 per cent 0_2 was again substituted for the N_2, and the muscle was allowed to recuperate for approximately 3 hours. It was then frozen, trimmed, and weighed, as described under Methods, and put in a glass vial in the deep freeze at -20°C. The other muscle was left in the refrigerator for about 2 days, mounted in the holder, and treated as control for the first, except that the period of anoxia was omitted. Free and total creatine were determined on both after the aged muscle had been frozen, trimmed, and weighed. Paired muscles were always analyzed in the same run. A standard curve of known creatine concentrations was determined each time.

The results for 6 pairs are tabulated in Table III. Another 3 pairs were not analyzed because the twitch tension of the aged muscles was < 50 per cent of the controls. The figures on another pair in which the twitch tension of the aged member had fallen to 62 per cent of the control are not included. In that particular pair the free Cr to CrP ratio of the control was 0.86 and

TABLE III

CREATINE-PHOSPHATE LEVELS AND CYTOCHROME *b* REACTIONS IN FRESH AND AGED TOAD SARTORII

	No. of hrs. aged	Concentration		Ratio FrCr/CrP	Oxidation of cytochrome *b* in response to										
					20 twitches no substrate			Pyruvate	10 twitches + substrate			20 twitches + substrate		
		Fr Cr	CrP		Supermax	Max	$t_{1/2}$ off		Supermax	Max	$t_{1/2}$ off	Supermax	Max	$t_{1/2}$ off
		mmoles/kg			*per cent*	*per cent*	*min.*	*per cent*	*per cent*	*per cent*	*min.*	*per cent*	*per cent*	*min.*
1a	—	8.8	20.7	0.43	4.1	−4.4	0.4*	−6.1	6.7	4.2	2.8	6.9	−0.6	1.5*
b	44	11.1	16.4	0.73	6.2	6.0	18.4	−16.2	8.9	6.8	4.5	10.7	8.5	6.3
2a	—	8.2	23.6	0.35	4.9	−5.9	0.3*	−8.4	2.6	—§	2.0	5.2	−2.2	0.9*
b	46	‡1.1	16.4	0.74	8.9	9.3	>30‡	−27.5	11.1	7.9	6.5	12.2	9.3	8.2
3a	—	10.5	21.1	0.50	4.0	−3.1	0.7*	−8.8	4.1	3.0	3.4	4.6	—§	2.9
b	49	20.2	12.0	1.68	13.4	9.9	16.1	−16.0	9.6	8.7	4.6	13.8	9.3	4.4
4a	—	3.3	9.8	0.34	6.4	−1.2	1.0*	−7.3	4.5	1.3	0.9	5.2	−1.6	1.1*
b	46	3.6	9.1	0.40	7.2	6.8	20.6	−19.9	6.4	—§	3.3	7.1	4.5	3.9
5a	—	3.2	10.0	0.32	1.3	—§	1.4	−6.8	4.7	3.4	3.0	6.1	1.2	2.0
b	48	3.8	10.3	0.37	5.8	5.8	26.2	−20.2	7.9	6.0	4.1	9.3	6.2	4.7
6a	—	7.9	30.8	0.26	3.1	−4.8	0.6*	−8.2	5.5	2.9	3.1	7.0	—§	2.5
b	47	8.9	28.8	0.31	11.3	11.0	>30‡	−32.1	5.8	4.2	5.7	7.5	5.5	6.9
Mean a	—	7.0	19.3	0.37	4.0	−3.2	(0.7)‖	−7.6	4.7	3.0	2.5	5.8	—**	(1.8)‖
b	46.7	10.0	15.5	0.71	8.8	8.1	20.3¶	−22.0	8.3	6.7	4.8	10.1	7.2	5.7

* Time to return to one-half supermaximal value. (Because the further response shows a reduction (a minimum actually) the $t_{1/2\ off}$ values of these responses are not directly comparable to the others.)

‡ Still more than 60 per cent oxidized 30 min. after activity.

§ No true maximum or minimum present, deflection declines monotonically.

‖ Averaged for both supermaximal and maximal values.

¶ Excluding muscles 2b and 6b.

** Qualitative differences preclude taking a mean.

that of the aged member, 2.60. The cytochrome responses of both were small and the response to substrate practically absent. The tension development of the aged members of the 6 pairs reported in Table III was better than 75 per cent of that of their fresh partners.

The most important figure, indicating the state of the ~ P stores, is the column of the ratio of the free creatine to creatine phosphate. The means are 0.37 and 0.71 for the fresh and aged specimens, respectively. Although at first sight the difference seems considerable, a *t*-test analysis shows that the probability is great that the two parameters belong to the same population ($p > 0.05$). (The difference in the means is largely due to one specimen only: muscle 3b.) The variation of the parameters of the cytochrome *b* response is so much greater that no statistical analysis is needed to show the differences between fresh and aged muscles. Perhaps the most significant measures are the differences in the maxima both before and after substrate addition. The long continued oxidation of cytochrome *b* in the aged muscles after 20 twitches is entirely absent in the fresh ones.

GLYCOLYSIS

Measurements on the glycolytic activity were performed under similar conditions. One member of a pair of toad sartorii was frozen 3 to 4 hours after excision, the other after a suitable aging period at the low temperature. In these experiments no pyruvate was added in order to forestall the formation of a high lactate level at zero time. The assay of the cytochrome *b* concentration by anoxia was omitted for the same reason. After the appropriate measurements had been made of the response of cytochrome *b* to twitches, the muscles were frozen and broken into 4 pieces with cooled forceps. These were weighed, after which 3 were allowed to thaw in a N_2 atmosphere and were refrozen after varying intervals. The lactic acid formed during the freeze-thaw contracture was then determined.

A difference in the time course of lactate formation was found between the fresh and aged samples. While the fresh ones showed a logarithmically decreasing rate, the aged ones produced lactate at a constant rate for at least 1 hour. For purposes of comparison, the rate of lactate formation of the fresh sample was plotted on a semilogarithmic scale and extrapolated to zero time. This initial rate can then be compared with the rate found in the aged member of the pair.

The results on 8 pairs of muscles are tabulated in Table IV. For comparison of the cytochrome *b* responses the $t_{1/2\ \mathrm{off}}$ values are given as the most reliable parameter in the absence of a determination of the cytochrome *b* content. The rates of lactate formation are generally not too widely scattered except for No. 6. In this pair the rates of both the fresh and the aged member

are approximately half those of the other pairs, while the responses of cytochrome *b* are quite in agreement with the others. No explanation of this anomalous behavior can be given. The ratio of the rates is, however, quite comparable with the other values.

TABLE IV

GLYCOLYTIC RATE AND CYTOCHROME *b* RESPONSE TO 10 TWITCHES IN FRESH AND AGED TOAD SARTORII

	Time aged	Lactic acid production	Ratio of the rates	Cytochrome *b* oxidation $t_{1/2\ off}$
	hrs.	*mmoles kg.*$^{-1}$ *hr.*$^{-1}$		*min.*
1a	—	204	5.9	7.4
b	43	34.8		25.4
2a	—	251	8.2	5.6
b	46	28.0		21.6
3a	—	215	5.4	—*
b	46	40.1		21.5
4a	—	210	2.1	6.0
b	48	98.9		18.4
5a	—	197	4.9	9.2
b	41	40.3		23.6
6a	—	102	5.7	7.5
b	44	17.8		>30
7a	—	227	9.1	4.0‡
b	42	24.9		>30
8a	—	204	1.9	—*
b	44	108		19.9
Mean a	—	201	5.4	6.6
b	44.5	49.1		21.7§

* Cytochrome *b* response not measurable with certainty.

‡ $t_{\frac{1}{2}\ off}$ of supermaximum. Cytochrome *b* became slightly reduced in later part of cycle.

§ Excluding muscles 6b and 7b.

It is clear that the capacity for lactic acid production is severely impaired in the aged muscles. This observation clarifies the responses of members of the respiratory chain. The aged muscles appear to suffer from a lack of oxidizable substrate, which is especially clear from Fig. 3. The defect is apparently located in the glycolytic chain.

A similar set of experiments, differing only in that the muscles were ground with sand, and lactic acid production was studied after the addition of various cofactors (DPN, ATP, and Mg^{++}), failed to reveal consistent differences between fresh and aged muscles. Although this type of experiment was not pursued further, it suggests that a lack of one of these cofactors, rather than a defect in the enzyme complement, is involved in this impairment of the glycolytic system.

V. *Measurement of Glycolysis in Intact Muscle*

It is possible to follow the time course and intensity of the glycolytic reaction by monitoring the changes in the pH (Lipmann and Meyerhof, 1930; Meyerhof, Möhle, and Schulz, 1932; Dubuisson, 1939; Distèche, 1960). With the present equipment this can be accomplished quite conveniently by measuring the color changes in an extracellular indicator. Two means exist for the transmission of the pH changes from the intracellular milieu: (*a*) diffusion of metabolites through the membrane, and (*b*) movements of CO_2 into and out of the cell in response to displacements of the intracellular carbonic acid equilibrium. Both would probably contribute.

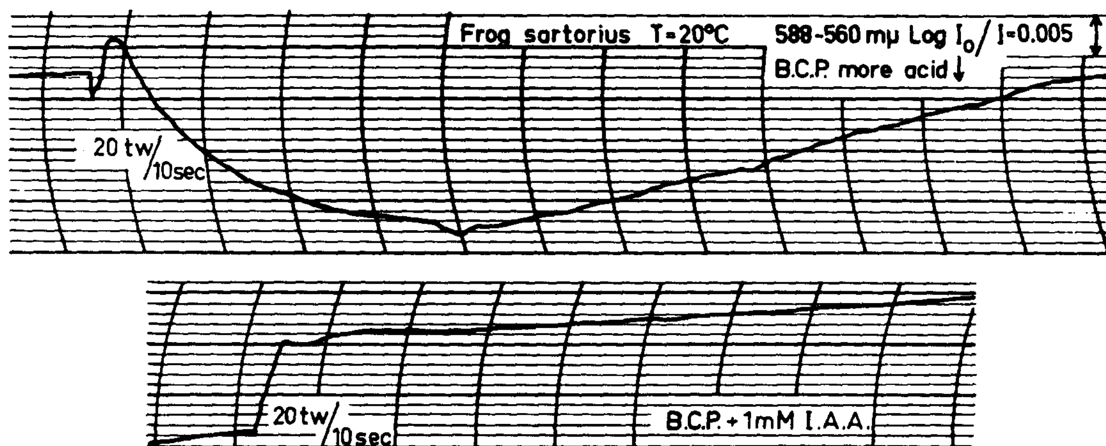

FIGURE 15. Intercellular pH changes caused by contractile activity in the absence and presence of iodoacetate; frog sartorius equilibrated with 0.004 per cent (*w/v*) brom cresol purple. Time scale is 1 minute per division.

A freshly excised and equilibrated muscle was mounted in the muscle holder and exposed to a Ringer's solution containing 0.004 per cent (*w/v*) brom cresol purple. After a period had been allowed for the penetration of the indicator into the intercellular space, the fluid was withdrawn to a level below the optical window, and the changes in the concentration of the basic form were measured at 588 to 560 or 588 to 570 mμ. The results are shown in the top record of Fig. 15 for a frog sartorius muscle. The main features of the trace are a rapid change to alkalinity followed by a protracted acidification.

The muscle was then equilibrated for 83 minutes with the same indicator solution to which iodoacetate was added to a level of 10^{-3} M. The bottom trace shows clearly that after this treatment the protracted acidification is missing. The acidification is, therefore, ascribed to the products of the glycolytic reaction, probably lactic acid. The early shift to alkalinity can best be con-

sidered as being caused by the breakdown of CrP. (See Meyerhof and Lohmann, 1928; Dubuisson, 1939.) Monitoring of cytochromes a and a_3 showed clearly that the formation of lactic acid took place without any trace of anoxia.

An indication of the minimal amount of activity necessary for the release of lactic acid can be gleaned from the time needed for the basic reaction to return to half its maximum value (*i.e.*, halfway to the initial base line). In the absence of lactic acid formation this would indicate a restoration of the CrP level and could be expected to increase with the number of twitches. When glycolysis is initiated by activity, a concomitant production of acid would, however, tend to cut this time shorter. In Fig. 16 the intensity and the $t_{1/2\ \mathrm{off}}$ of the basic reaction are plotted as a function of the number of twitches in a frog sartorius. The time was measured from the last stimulus. The twitches

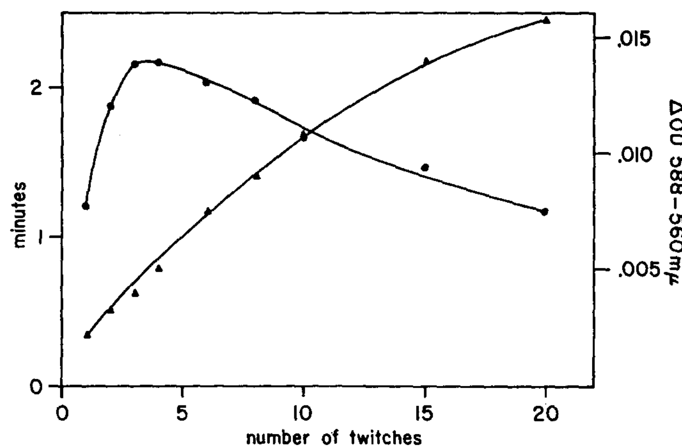

FIGURE 16. Intensity and time course of the basic reaction in response to varying numbers of twitches; frog sartorius, 18°C, no substrate added. Solid circles, $t_{1/2\ \mathrm{off}}$, left ordinate; solid triangles, amount of deflection in the basic direction, right ordinate.

were equally spaced over a 20 second interval; *i.e.*, the first twitch was given at −20 seconds.

The basic reaction (CrP breakdown) increases directly with the number of twitches. The tendency toward a saturation of the response at the higher numbers is probably a reflection of an earlier onset of the acidification by glycolysis. The variation of the $t_{1/2\ \mathrm{off}}$ shows clearly that after 4 twitches an acidification must be superimposed on the response. In all probability this occurs even after 2 contractions, as can be seen from the non-proportionality of the $t_{1/2\ \mathrm{off}}$ figures for 1 and 2 contractions.

Similar experiments with fresh toad sartorii produced very much the same results. After approximately 5 twitches the acidification response was clear, while probably even fewer twitches produced an acidification. The acidification reaction was absent in aged muscles. In Fig. 17 the cytochrome b and

pH responses of a muscle before and after aging are shown in response to 10 contractions. The two lower curves indicate the responses of the fresh muscle, and a slight but definite pH fall concomitant with a slight reduction of cytochrome *b* is seen. After approximately 40 hours of aging at 0°C the cytochrome *b* response typically showed a lack of substrate, while the pH effect remained a basic one. It seems clear that, after the aging treatment, the muscle fails to produce lactic acid in response to the activity imposed upon it.

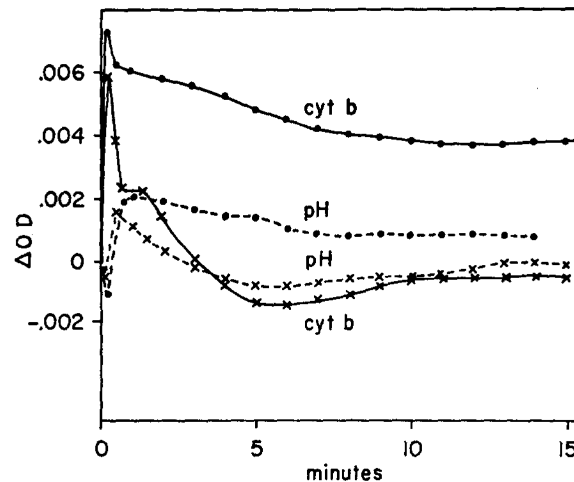

FIGURE 17. Cytochrome *b* cycles and concomitant pH changes in a toad sartorius muscle in response to 20 twitches in 20 seconds; 22°C, no substrate added. Crosses, 4 hours after excision (*i.e.*, fresh); circles, after 40 hours at 0°C. Cytochrome *b* was measured at 430 − 410 mμ, the pH changes by means of the color changes of 0.004 per cent (*w/v*) brom cresol purple at 588 − 560 mμ; upward deflections signify oxidation and alkalinization, respectively.

DISCUSSION

The reactions of the isolated muscle mitochondria described in this paper are consistent with the results described for other mitochondria (Chance and Williams, 1955; Chance and Baltscheffsky, 1958), provided the lack of respiratory control in the presence of ATP is taken into account. In addition it was shown that they do not respond to addition of carnosine, creatine, AMP, hypoxanthine, inosine, or Mg^{++} and only partially and erratically to Ca^{++}. All of these do or can be expected to change in concentration as a result of contractile activity. Of the naturally present compounds only an increase in ADP produced reasonable increases in respiration.

In the previous paper of this series it was shown that in resting muscles cytochrome *b* is considerably reduced and it was surmised that the same is true for DPN. The changes in the steady states resulting from addition of substrate or from contractile activity can now be compared with the findings

on the isolated muscle mitochondria and with the more complete observations of Chance and Williams (1955) and Chance and Baltscheffsky (1958).

In fresh muscles the result of a few twitches is the oxidation-reduction cycle of DPNH, an oxidation of cytochrome *b*, and a reduction of cytochrome *c*. More activity usually resulted in a reduction of all three components. In aged muscles, in which the glycolytic chain was shown to be greatly impaired, DPNH and cytochrome *b* both continue to become more oxidized with increasing amounts of activity. These observations, coupled with the observed increase in reduction of cytochrome *b* and DPN upon the addition of lactate or pyruvate, noted in the fresh as well as in the aged muscles, lead to the conclusion that the mitochondria in the fresh, intact muscles examined here do not have substrates available in excess amounts to cover the need for the increased respiration following activity. Viewed together with the observation that the responses of DPNH and cytochrome *b* to contractile activity become more prominent after the increased reduction of these components upon the addition of a suitable substrate, it must be concluded that the mitochondria in the intact skeletal muscle tissue are not in a condition comparable to state 4 of the Chance and Williams scheme. It appears rather that the condition more closely resembles their state 1, in which the ADP level is also limiting the rate of oxygen uptake, but in which the substrate level is low: so low, as a matter of fact, that neither DPN nor cytochrome *b* attains its true state 4 reduction level.

These conclusions imply that after very limited contractile activity, and perhaps after only 1 twitch, more substrate is made available. Lack of a reduction of cytochromes *a* and a_3 shows that this can occur under completely aerobic conditions. This seems, at first, in disagreement with the classical concepts about muscle metabolism, according to which lactic acid production does not occur after mild exercise under aerobic conditions. These conclusions rested, however, on chemical measurements of, perhaps, insufficient sensitivity. The present observations show that the lactic acid concentration rises temporarily only. After a series of as many as 30 twitches the original pH is restored at the end of the recovery period, provided no part of the muscle becomes anoxic. When anoxia does occur, the recovery to the normal pH is usually greatly protracted. The effects of tetanic stimulation, however, which causes a long lasting reduction of DPN, cytochrome *b*, and cytochrome *c*, would suggest an accumulation of substrate greater than can be metabolized quickly. Under those circumstances an accumulation of lactic acid must certainly take place.

It is a tribute to the scientific acumen and the remarkable powers of observation of Professor Keilin that he noted the same effect at an early stage in his description of the cytochromes (1925). He observed a reduction of these components in insect flight muscles when the animal commenced beating its

wings. This fact still stands, even if the occurrence of anoxia was not completely excluded in his preparations.

ADP As the Limiting Factor for the Respiration

So far the responses of the respiratory carriers have tacitly been assumed to be caused by increases in the ADP level. They may, however, also be caused by a number of other agents. Most of these fall in the class of uncouplers, substances which disengage respiration from the control of phosphorylating reactions. In view of the barren nature of this type of stimulation of oxygen uptake, and also, because those compounds generally do not occur in tissues, they may be written off as causative agents in the observed responses. Calcium ions do occur, however, in the muscle and appear to play an important role in the contractile and activation mechanisms (Weber and Portzehl, 1954; Bianchi and Shanes, 1959). This ion, however, also has an uncoupling effect on oxidative metabolism (Potter, 1947; Lehninger, 1949; Slater and Cleland, 1953) and thereby can justifiably be ruled out as the active agent.

In the present experiments with isolated mitochondria no stimulation of respiration was noted with a series of metabolites known or suggested to be raised in concentration by contractile activity (creatine, carnosine, AMP, hypoxanthine, inosine, Mg^{++}). Inosine is of special interest. This compound was shown by Parnas (1929) to be formed in response to contractile activity. Recently this was corroborated by the studies of Wajzer *et al.* (1952, 1958). The possibility that this would constitute the primary energy-donating reaction in muscular contraction appears to be ruled out by the extensive experimentation of Fleckenstein *et al.* (1954) and of Mommaerts (1955).

In the process of oxidative phosphorylation phosphate ions, as well as ADP, can be the rate-limiting substances under certain, specific conditions. This can occur when the other of the two is present in excess. The process of stimulation of the metabolism is, however, much more efficiently performed by ADP than by P_i. The first has a K_M of 2 to 5 $\times$ 10^{-5} M, while the latter produces the same effect at 5 $\times$ 10^{-4} M in rat liver mitochondria (Chance and Williams, 1955) or at 2 $\times$ 10^{-5} M in pigeon heart mitochondria (Chance and Hagihara, 1961). In the present experiments with toad muscle mitochondria, the K_M's for ADP and P_i were found to be 46 and 710 μM, respectively.

The most recent analyses find levels of 0.25 mmole/kg ADP in frog sartorii with indications that the level actually must be about 0.10 mmole/kg (Seraydarian, Mommaerts, and Wallner, 1962). The P_i concentration appears to be 1 mmole/kg in thoroughly cold-adapted specimens (Seraydarian, Mommaerts, Wallner, and Guillory, 1961) but closer to 2 to 3 mmole/kg in the muscles of animals kept at room temperature such as ours (Mommaerts and Seraydarian, personal communication). These concentrations are above those needed for maximal respiration, and it can only be concluded that the

intact tissue does not in reality contain such high concentrations of both substances, or, at least, that such high levels are not available to the mitochondria. If such levels were present, respiration would always proceed at its maximal rate, especially after addition of diffusible substrates.

It is possible to calculate the levels of ADP and P_i in the intracellular milieu of the resting muscle from the affinity determinations of isolated mitochondria (Fig. 2) and from the oxygen consumption rates of the intact muscles. The rate of respiration in excised resting frog muscles is stimulated approximately 100 per cent by the addition of lactate (Meyerhof and Lohmann, 1926; Smillie and Manery, 1960; Gourley and Fisher, personal communication, 1962). A considerable fraction of this increased O_2 uptake is apparently required for the glycogen synthesis, as revealed by the addition of iodoacetic acid (IAA) (Gourley and Fisher, personal communication, 1962). In the presence of both substrate and inhibitor the metabolic rate is only 50 per cent higher than in the normal resting state. This condition represents the closest approximation to a resting state with excess substrate (state 4).

During long lasting contractile activity the respiration of frog muscles will increase approximately sevenfold over the resting rate in the absence of lactate and IAA (Meyerhof and Schulz, 1927; D. K. Hill, 1940). The maximally active respiration (state 3) proceeds, therefore, at 4.7 times the resting rate in the presence of excess substrate (state 4). Extrapolation of the ADP affinity curve to 0 respiratory rate (at -30 μM ADP on the scale shown in Fig. 2) provides a new origin.[4] The extrapolated curve gives a coordinate point at 26 μM ADP (-4 on the scale shown in Fig. 2) from this new origin for a respiratory rate of (1/4.7) times the maximal one. This means, therefore, an ADP concentration of 26 μM in the frog muscles in the resting steady state. A similar calculation for P_i produces a figure of approximately 350 μM for the resting level. In view of the recent results showing an increase of P_i of 0.3 mmole/kg per twitch (Carlson and Siger, 1960; see also Cain and Davies, 1962; Mommaerts, Seraydarian, and Marechal, 1962) and the fact that 1 twitch, which would raise the P_i from 350 to 650 μmoles, does not nearly result in a half-maximal rate of oxygen consumption, it can be concluded that the intracellular concentration of ADP and not of P_i is limiting[5]. It can also be concluded that the ADP level in the resting muscle must be close to

[4] The designation of the value 0 μM ADP to this point makes the reasonable and as yet uncontradicted assumption that in the intracellular milieu the mitochondria are completely coupled; *i.e.*, that no respiration takes place without concomitant oxidative phosphorylation. The respiratory rate of 23 per cent V_{max} observed in isolated mitochondria in the absence of added ADP is considered to be the result of uncoupling due to damage incurred during the isolation procedure.
[5] It was observed that twitching raised the substrate level only to an extent approximately equal to the demands for substrates for oxidative phosphorylation. The amount of P_i used up in glycolytic formation of ATP is, therefore, negligible for these calculations for recently excised muscles and even more so for the aged toad sartorii.

the 26 μM figure derived here. The higher concentrations found by direct analysis are probably related to an uncontrolled breakdown of ATP during such analyses (see Seraydarian, Mommaerts, and Wallner, 1962). Recovery studies of added ATP appear to rule out a breakdown of this compound in the free ionic form but do not eliminate the possibility of a hydrolysis of fractions adsorbed to various intracellular constituents.

The existence of very low levels of ADP can also be surmised from studies by Cobey and Handler (1953) on the turnover of P^{32} in AMP, ADP, and ATP in liver, kidney, and muscle tissue of cats. These authors showed that this turnover is by far the smallest in muscle. In view of the approximately equal levels of myokinase (Kotel'nikova, 1949), as well as of ATP and AMP in these tissues, it must be concluded that the ADP levels of muscle are much smaller than shown by direct analysis. An estimate based upon the data of Cobey and Handler would set the level of ADP in muscle in the micromolar range in agreement with the data presented in this article.

Intracellular ADP Increases per Twitch

The response of the mitochondria within the intact muscle to contractile activity has been shown to be qualitatively in complete agreement with the response of isolated mitochondria to the addition of ADP in the presence of P_i and substrate. By a method of elimination of other candidates, it was concluded that the probability is great that the effects are truly responses to ADP. There remains a quantitative consideration of the amount of ADP arriving at the mitochondria.

The values for the P_i increase after 1 twitch (Carlson and Siger, 1960) or after a short tetanic contraction (Cain and Davies, 1962 *a*; Mommaerts *et al.*, 1962) agree closely with the amount of $\sim$ P broken down by such contractions as calculated from the existing work and heat data. Very recently Cain and Davies (1962 *b*) and Infante and Davies (1962) have reported that they can show a similar amount of ATP breakdown in muscles treated with dinitrofluorobenzene (DNFB). They ascribed their success to an inhibition of creatine phosphate transphosphorylase (CrPTase) by this compound. Although verification of the specificity of this inhibitor must still be forthcoming, a task which may be difficult in view of the non-specific nature of this inhibitor and its disturbing effects on oxidative metabolism (Chance, Jöbsis, and Mauriello, data in preparation), it is nevertheless highly significant that in the presence of DNFB 0.4 mmole of ATP/kg appears to be broken down in 1 twitch. For the following calculations, therefore, a breakdown of 0.3 to 0.4 mmole of high energy phosphate/kg per twitch will be used.

Many enzyme systems that have the effect of buffering any ADP concentration changes are present in the sarcoplasm. Most prominent in this respect are, perhaps, the myokinase (MKase) and CrPTase systems, which would

partly restore the ATP level, by transferring $\sim$ P to ADP from ADP and creatine phosphate (CrP), respectively. The glycolytic system should also be considered as a possible candidate.

Although glycolysis is generally thought to proceed slowly or perhaps not at all under aerobic conditions, the measurements of the pH changes and the difference between the fresh muscles and those in which this system is apparently impaired by aging suggest otherwise. In a series of twitches the amount of extra substrate appears to be close to the amount needed for oxidative recovery. The amount of ADP converted to ATP by the glycolytic system would then be an insignificant fraction only. The response to a tetanus, however, overshoots the needs of the respiratory chain greatly, as shown by the long lasting reductions of some of the carriers and the intense acidification. Estimation of the initial rate of lactic acid production in the freeze-thaw contractures of toad sartorii yields an average of about 200 mmoles kg^{-1} $hr.^{-1}$. Oxygen consumption studies, though less complete, lead to an average rate of 610 ml kg^{-1} $hr.^{-1}$ for maximal respiration after contractile activity. Considering an ATP yield of 1.5 moles per mole of lactic acid formed and a maximum of 3.2 moles of ATP for each mole of atomic oxygen consumed, we arrive at rates of 300 and 174, respectively, for the two systems. Thus the glycolytic system of the toad sartorius has the inherent capacity to regenerate high energy phosphate at a rate 1.7 times higher than the oxidative phosphorylation system. Glycolysis should, at least after a tetanus, be able to lower the ADP concentration at a rate great enough to have an important effect on the level available to the mitochondria. The observation that contractile activity results in much larger oxidation responses of cytochrome *b* and DPNH in the aged toad sartorius agrees well with the finding that the capacity for glycolysis is decreased to one-fourth by the aging treatment.

The role of creatine phosphate and the CrPTase must also be of great importance in the control of intracellular ADP levels. No good values of the turnover number of this enzyme under intracellular conditions are available. Carlson and Siger (1960), however, have put an upper limit on the time constant of this reaction, assuming pseudomonomolecular kinetics. They have come to the conclusion that at 0°C this constant is smaller than 30 seconds. With the assumption of the rather low Q_{10} value of 2.0, we would arrive at 7.5 seconds or less at 20°C, the approximate temperature at which most of the present experiments were performed. This being only an upper limit for complete equilibration, it is clear that the CrPTase system will strongly influence the ADP level available to the mitochondria. The main effect would be a rapid and drastic lowering of the ADP concentration after and probably even during a contraction.

It is possible to arrive at an estimate of the amount of ADP formed per twitch from the concentration of this substance needed to stimulate respira-

tion in isolated mitochondria. The K_M of ADP for the stimulation of O_2 uptake is 4.6×10^{-5} M, while for P_i it is 7.1×10^{-4} M. In similar experiments in which the response of DPNH in isolated liver mitochondria was used as an end point, Chance and Connelly (1957) have found the K_M of ADP to be 5×10^{-5} M (see also Chance, 1959). For extrapolation to intracellular mitochondria, any of the responses of aged muscles supplied with substrate can be taken. Usually 3 to 6 twitches are needed to attain half-maximal oxidation of DPNH under such conditions. This means that the level of ADP must have been raised from 26 to 46 μM. Thus, we arrive at a figure of 3.3 to 6.6×10^{-6} mole/kg for the amount of ADP produced per twitch, as measured by the DPNH response. This corresponds to an average of 5×10^{-3} mmole per kg. The amount observed in the intact muscle is, therefore, only about 1.6 per cent of the expected value. In view of the interference by lactate production, only the early response of cytochrome *b* ($P_{supermax}$) in fresh muscles can be used for similar calculations. This provides a figure of 2 to 4 per cent of expectation.

This discrepancy between the expected ATP breakdown and the observed increase of ADP, already noted by Chance and Connelly (1957), has erroneously been taken by some (see for instance Nekhorocheff, 1959) as support for the view that ATP is not directly involved in the reaction supplying energy to the contractile machinery (Fleckenstein *et al.*, 1954; Davies *et al.*, 1959; Mommaerts, 1954, 1955). The following considerations throw doubt upon the correctness of that conclusion.

The data gained from analysis are sufficient to determine the equilibrium constant, $K = \frac{[\text{CrP}]\cdot[\text{ATP}]}{[\text{CrP}]\cdot[\text{ADP}]}$, for the CrPTase system. For resting frog muscles a number of recent studies (Fleckenstein *et al.*, 1954; Carlson and Siger, 1959; see also Davies *et al.*, 1959) have found concentrations of ATP of 2 to 3 mmoles/kg. This level will be assumed to exist also in the toad sartorii. A level of 26 μmoles/kg will be used for the ADP concentration.[6] For the fresh and aged toad sartorii separate calculations should perhaps be made based on the analysis of Cr and CrP presented here, although the differences are small and probably not significant. Assuming a breakdown of 0.3 to 0.4 mmole of ATP/kg, we can calculate that when equilibrium is reestablished the net increase of ADP is approximately 2 and 3 μmoles/kg per twitch for the fresh and aged muscles, respectively.

These values are, of course, only approximations. The effect of the glycolysis can probably be neglected in this calculation, but the myokinase activity is actually an unknown factor. The CrPTase system is, however, almost cer-

[6] The assumption of 0.1 to 0.2 mmole ADP/kg as found by direct analysis would raise the amount of ADP to be found after 1 twitch by a factor of 3 to 5.

tainly of overbearing importance in view of the absence of increases in AMP concentration after limited numbers of twitches (Fleckenstein *et al.*, 1954; Mommaerts, 1954, 1955). A new equilibrium must have become established within a few seconds after a twitch as was estimated above and probably much faster. The experiments with muscles poisoned with DNFB (Cain and Davies, 1962 *b*; Infante and Davies, 1962) indicate that in untreated muscles the CrPTase system may not even be too far off equilibrium during the contraction. Thus, the CrPTase system reacts sufficiently rapidly to come to equilibrium in a short time compared with the duration of the recovery respiration.

It is evident that the discrepancy between 2 to 3 μmoles of ADP calculated from the CrPTase equilibrium and the level of 5 μmoles deduced from the observations on the respiratory chain is sufficiently small to throw doubt on the recent conclusions that ATP is not the energy donor for the mechanical energy and heat expended in 1 twitch (Fleckenstein *et al.*, 1954; Mommaerts, 1954, 1955; Davies *et al.*, 1959; Cain and Davies, 1962 *a*). The opposite conclusion appears to be more valid.

This study was supported by a grant from the Muscular Dystrophy Associations of America, Inc. Many thanks are due Dr. W. F. H. M. Mommaerts and the members of his laboratory for helpful criticism of this paper and for previews of some of their as yet unpublished work.
Received for publication, September 26, 1962.

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