Oxidative and Glycolytic Recovery Metabolism in Muscle

Fluorometric observations on their relative contributions

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ABSTRACT The average degree of reduction of mitochondrial NAD has been measured in the intact toad sartorius by a fluorometric technique. It has been shown that cytoplasmic NADH does not interfere materially with these measurements. The percentage reduction of this respiratory coenzyme has been determined in a number of physiological steady states which are well correlated with fluorometrically determined levels of NADH in suspensions of mitochondria from the hind leg musculature of the toad. In addition, these findings are closely comparable to similar, spectrophotometric measurements on mitochondria from other sources. In the presence of an adequate O_2 level a single twitch produces a decrease in fluorescence from the resting steady state which is followed by a slow return to the base line condition. This cycle indicates the intensity and the time course of the oxidative recovery metabolism. The area under this curve is directly related to the number of twitches up to three or four. Greater activity produces a curtailment of oxidative recovery due to glycolysis. In the presence of iodoacetate the linear relation holds for five to seven twitches. At still higher levels of activity a curtailment of the change in NAD level sets in, probably due to the removal of AMP by catabolic reactions.

INTRODUCTION

Spectrophotometric observations on the steady-state oxidation-reduction levels of members of the respiratory chain in intact muscle showed that changes in these levels can be used for monitoring the recovery metabolism (Chance and Connelly, 1957; Jöbsis, 1963 *b*; Chance and Weber, 1963). Previously, the work of Chance and Williams (1955) had established that the rate of oxygen

uptake by isolated mitochondria is closely correlated with the percentage of reduction of the cytochromes, flavoproteins, and NADH[1] via which reducing equivalents flow from substrates to oxygen. The rates of mitochondrial respiration and oxidative phosphorylation are also highly dependent on the concentration of ADP. Conversely, the discrete levels of reduction of the members of the respiratory chain can serve as indicators of the changes in these rates due to an increase in the ADP concentration. These observations were extended to, and verified in, living tissues. By the application of spectrophotometric techniques it was, therefore, possible to monitor the changing levels of ADP in muscles during and after contractile activity. (For a review of this approach see Jöbsis, 1964 *a*.)

The studies on toad and frog muscles showed, however, that in the freshly excised preparations the oxidation-reduction levels were complexly related to the amount of contractile activity (Jöbsis, 1963 *b*). After 48 hr at 0°C these complexities were minimized due to the inactivation of the glycolytic system. It was then possible to analyze the spectrophotometric signals obtained from NADH, cytochrome *b*, and cytochrome *c* in the frame work provided by the studies with isolated mitochondria, and to show, qualitatively, that the complexities of freshly excised muscles arose from increased levels of mitochondrial substrate due to stimulation of glycolysis. It is known that increased levels of substrates induce a greater reduction of the respiratory chain, whereas a higher ADP concentration produces the opposite effect in cytochrome *b* and NAD. The spectrophotometric signals reflected these two effects by diminished or diphasic responses to contractile activity.

In 1959 Chance and Jöbsis showed that the reduction level of NAD in intact tissue (excised muscle) can be monitored fluorometrically. This technique hinges on the fact that light of the 340 mμ region elicits fluorescence from the molecule when reduced (NADH) but not when oxidized (NAD^+). This original observation was subsequently verified by spectral measurements of the emission and was extended to tissue *in situ* with intact blood supply (Chance, Cohen, Jöbsis, and Schoener, 1962).

In the present paper the applications and limitations of the fluorometric method for excised muscle tissue are examined more quantitatively. The technique is then applied to the investigation of the relative extent of the participation of oxidative phosphorylation and glycolysis in the recovery metabolism after contractile activity.

[1] The following abbreviations are used: NAD = nicotinamide adenine dinucleotide (NADH the reduced and NAD^+ the oxidized form), NADP = nicotinamide adenine dinucleotide phosphate, (NAD = DPN and NADP = TPN in the previous nomenclature). ATP = adenosine triphosphate, ADP = adenosine diphosphate, P_i = inorganic phosphate, ~P = high energy phosphate bond, IAA = iodoacetic acid.

METHODS

I. *Instrumentation*

Most experiments were performed with a new differential fluorometer designed for the present type of experimentation (Jöbsis, Legallais, and O'Connor, 1966). The optics are arranged for surface detection; i.e., fluorescence is measured from the illuminated surface of the sample. The excitation light is obtained from a high pressure mercury arc lamp equipped with a 365 mμ filter. The secondary filter eliminates all wave lengths below 405 mμ and thereby transmits the tissue fluorescence which has a maximum at approximately 465 mμ. The instrument compares the intensity of fluorescence of the sample (the muscle) with that of a stable reference material (a small square of paper). This is accomplished by switching the excitation light beam alternately between the reference and the sample. The great instability of the arc lamp was compensated for by the introduction of feedback to maintain the reference signal at constant intensity. For this the reference signal is isolated and, after suitable amplification, is made to control the grid of a pass-tube in series with the high voltage power supply to the photomultiplier. This high voltage level is clamped and thereby maintained constant during the subsequent illumination of the sample. The difference in fluorescence between the muscle and the reference material is recorded as a function of time.

With a second piece of paper substituted for the muscle in the sample beam the instrument generally exhibits a peak to peak noise level of 0.1% (time constant = 2.8 sec) and no long term drift (20–30 min) is observed. With a well equilibrated, resting muscle in place short term noise is 0.2% and the drift over a 15 min period is also about 0.2%. Most of the peak to peak noise level in excess of 0.1%, observed with a biological sample, must be assigned to disturbances introduced by bubbling the solution with oxygen. The difference in long term drift is ascribed to metabolic fluctuation in the muscle.

For a limited number of experiments a double beam spectrophotometer was utilized for spectrophotometric measurements on NADH in the manner previously described (Jöbsis, 1963 *b*).

The muscle was held vertically in a cuvette provided with flow channels for tesm perature control. Illumination and detection were from the deep surface of the muclein order to avoid the fluorescence of the thick epimysium which covers the fibers on the superficial, skin side. In most experiments the pelvic tendon was clamped between two small, stainless steel bars which formed the lower attachment. The knee tendon was attached to a lever via a minimum (1-2 mm loop) of cotton thread (No. 8), stainless steel hooks, and a light silver chain. The lever consisted of an aluminum bar, provided with a short axle of stainless steel, which turned in two ball-bearing sockets in a substantial block of aluminum. The arm of the lever to which the muscle was attached bore two semiconductor strain gauges for the measurement of tension. They were connected to contacts on the stand with fine, coiled wires. For registration of displacement, a second tension-transducing system was coupled to the lever via a soft

spring close to the fulcrum (lever ratio 44:1). The tension was balanced by a counterspring on the other side of the fulcrum. Thus motion was transformed into tension with a negligible increase of force output by the muscle (approximately 200 mg per cm shortening). The mechanical performance of the muscle was registered by an Offner recorder (Offner Division, Beckman Instruments, Inc., Schiller Park, Ill.) with fast time response. The frequency of the isometric system was about 75 cps without attenuation and that of the isotonic system approximately 60 cps.

Stimulation of the muscle was via two long, bar electrodes parallel in the solution, and about 3 mm away from the edges of the muscle. This constituted, in essence, a "massive electrode" system for homogeneous, transverse field stimulation (Brown and Sichel, 1936). Stimuli were rectangular pulses of 1-2 msec duration (depending on the temperature) and at a voltage about twice that for a maximum twitch response. These were generated by an American Electronic Laboratories (Colmar, Pa.) Stimulator Model 104 A, the output of which was matched to the low impedance of the electrodes via an emitter follower circuit.

II. *Biological Materials*

Mitochondria isolated from the hind leg muscles of the toad and from rat kidneys were studied fluorometrically in addition to excised, sartorius muscles of the toad (*Bufo marinus*).

Mitochondria were isolated from the hind leg musculature of toads by a variation of the proteinase method (Chance and Hagihara, 1960). In this procedure the resistance of the tissue to homogenization is greatly decreased by previous treatment with a proteinase of bacterial derivation. The toad was killed by decapitation and the muscle tissue of the leg was dissected off and finely chopped with scissors. After a wash with the isolation medium [mannitol 225 mM; sucrose 75 mM; EDTA (ethylenediaminetetraacetic acid) 0.5 mM; albumin 2 mg per ml; pH adjusted to 7.4 with Tris (hydroxymethyl) aminomethane], a 20 ml aliquot of this medium was added which in addition contained 100 mg $KHCO_3$ and 40 mg proteinase. The enzyme reaction was allowed to proceed for 10 min at 0°C under continuous dicing with the scissors. The brei was homogenized with a Potter-Elvehjem homogenizer with especially large clearance using only two passes at approximately 250 rpm. The proteinase reaction was then continued for another 5 min after which the same homogenization procedure was repeated. The homogenate was made up to approximately 150 ml, strained through plastic gauze, and centrifuged in a Servall refrigerated centrifuge for 5 min at 500 × *g*.

The supernatant of this spin contains some mitochondria but the yield is low and they are contaminated with some unidentified black particles. Furthermore, the quality of this preparation is low as gauged by the respiratory control ratio. This fraction of mitochondria was, therefore, discarded.

The precipitate of the 500 × *g* –5 min spin was resuspended and homogenized more vigorously for 10 min by passing the pestle up and down by hand. The straining procedure was repeated and the precipitate coming down at 5000 × *g* for 10 min from the supernatant of a clearing spin at 500 × *g* for 5 min was used. The precipitate contained approximately 15 mg of protein and was diluted to 2 ml.

For the rat kidney mitochondria essentially the same procedure was followed except that a rehomogenization of the first precipitate at 500 × *g* was unnecessary. The supernatant of that spin was centrifuged at 10,000 *g* for 10 min and then resuspended and spun down again for some additional purification.

For the determination of the O_2 uptake and for the fluorometric measurements, the preparation was diluted with the reaction medium (sucrose 234 mM; KCl 11 mM; EDTA 0.2 mM; Tris-HCl 11 mM; Na-PO_4 buffer 22 mM; pH 7.4). Respiratory rates were measured polarographically in a closed cuvette for which 50 μl of the preparation described above were added to 0.95 ml of this medium. In the fluorometric experiments 0.2 to 0.3 ml of the preparation was used per 5 ml of reaction medium. Anoxia was prevented by playing a jet of 100% O_2 directly on the surface of the suspension with such force that a depression was formed without the occurrence of bubbles. In addition to sufficient oxygenation this also provided for very efficient stirring.

For the measurements on intact muscles sartorii of the tropical toad (*Bufo marinus*) were used. After decapitation, the hind quarters were perfused via the descending aorta with Ringer's solution (113 mM NaCl; 5 mM KCl; 2 mM $CaCl_2$; 2 mM phosphate buffer at pH 7.0). After careful excision of the sartorii, the muscles were treated in one of three standard ways. They were either used immediately ("fresh") or they were equilibrated overnight (or longer) in Ringer's solution with or without 10 mM pyruvate at 10°C. During this treatment the muscles were without constraint in a small flask with continuous bubbling of 100% O_2. In general, treatment of the muscles was identical with the method previously described (Jöbsis, 1963 *a*) except that previous administration of heparin for the prevention of blood clots was found to be unnecessary. Before mounting, the muscle was inspected under a dissecting microscope and all adhering debris was removed. These small fragments can constitute an annoying source of noise when they become detached and are carried along in the circulating Ringer solution.

RESULTS

I. *Experiments with Isolated Mitochondria*

Polarographic and fluorometric experiments were performed with mitochondria isolated from rat kidney and from hind leg musculature of the toad, partly for the investigation of the quantitative aspects of the fluorometry of NADH and partly for comparison with the fluorescence cycles obtained from intact muscles.

The mitochondria, isolated by the proteinase method described above, usually were of much better quality than those described previously (Jöbsis, 1963 *b*). They exhibited a respiratory control ratio:

$$\frac{\text{Rate of } O_2 \text{ uptake in presence of ADP}}{\text{Rate of } O_2 \text{ uptake in absence of ADP}} = \frac{\text{state 3 rate}}{\text{state 4 rate}}$$

ranging from 4 to 7, as assayed with the oxygen electrode technique (see Chance and Williams, 1955). This figure compares satisfactorily with that for standard preparations, such as rat liver mitochondria.

STEADY STATES IN SUSPENDED MITOCHONDRIA

In their original description of the oxidation-reduction levels of members of the respiratory chain, Chance and Williams (1955) were able to draw up a relatively simple scheme of five easily recognizable steady states. We attempted to identify these states fluorometrically in rat kindey and toad muscle mitochondria at two different temperatures (12 and 24°C). In Table I the results are compared with the spectrophotometric data of Chance and Williams in an

TABLE I

METABOLIC STATES OF ISOLATED MITOCHONDRIA. LEVELS OF FLUORESCENCE AND NAD REDUCTION

State		1	2	3	4	5
Designation		Endogenous	Starved	Active	Resting	Anoxic
Respiratory rate		Slow	~0	Fast	Slow	0
Rate limitation		ADP	Substrate	Electron-transfer	ADP	O_2
Fluorescence signal (State 5–state 2) = 100%						
			%	%	%	%
R. K. M. 24–26°C (9 preparations)		Variable	0	51	89	100
T. M. M. 24–26°C (5 preparations)		Variable	0	47	85	100
T. M. M. 11–13°C (5 preparations)		Variable	0	42	88	100
NAD reduction level measured by spectrophotometry*						
			%	%	%	%
Mammalian liver M. 23°C		Variable	0	50–60	~100	100

R.K.M., rat kidney mitochondria; T.M.M., toad muscle mitochondria.
* From Chance and Williams (1955 *b*)

effort to verify whether some or any of the fluorescence changes can be assigned to oxidations and reductions of the respiratory NAD.

In these experiments aliquots of the stock preparation of mitochondria were added to the suspension medium in the muscle cuvette until the fluorescence signal was approximately equal to that for intact muscles. The steady states were obtained in very much the same manner as in the original spectrophotometric experiments. State 1 is the condition found to exist upon the introduction of the mitochondria into the suspension medium. The substrate-depleted state (state 2) was obtained by adding ADP to the suspension until the fluorescence had decreased to a stable, low value, due to the depletion of

the endogenous substrate. At this point respiratory chain NADH is considered to be completely oxidized. The addition of substrate to such a suspension produces a maximum rate of O_2 uptake and oxidative phosphorylation, which is designated active (or activity) respiration (state 3). The velocity of the reaction in state 3 is limited only by the maximum reaction rate of the enzymatic system. Eventually all ADP will be transformed into ATP, whereupon the rate of respiration decreases and the level of NAD reduction rises. Now the respiratory rate is low, due to a lack of phosphate acceptor for oxidative phosphorylation. This is referred to as the resting metabolic condition (state 4). State 3 can be induced again by the addition of ADP. This is accompanied by an oxidation of NADH, the extent of which shows a typical saturation effect with the amount of ADP added. The state 3 level reported in Table I is that found for extrapolation to infinite ADP concentration (see Fig. 2). Upon the depletion of the ADP, respiration decelerates again and the fluorescence trace returns to the level in the resting condition (state 4). Finally an increased level of fluorescence (state 5) can be obtained either by adding cyanide or by allowing the mitochondria to consume all available oxygen (i.e. to become anoxic).

The percentage levels of reduction quoted in Table I, are related to the total difference in fluorescence which is obtained between the starved and anoxic states. In state 2 approximately 60 and 70% of the state 4 fluorescence still remains in the rat kidney and toad muscle mitochondria, respectively. This originates from other materials and from fractions of NAD or NADP not directly involved in the respiratory chain.[2] Perusal of Table I shows that the steady-state conditions measured spectrophotometrically and fluorometrically agree well in all points except for the further increase in fluorescence upon the state 4 to state 5 transition. In their original work, Chance and Williams found no further increase whereas in the present experiments an increase of 10 to 15% is evident. This discrepancy will be examined further in the Discussion.

In 1961 Chance and Hollunger described a second fraction of mitochondrial NAD which is reduced by succinate via a flavoprotein rather than by NAD-linked substrates. α-Glycerophosphate, another flavoprotein-linked substrate, is capable of doing the same to a limited extent. Since the free energy content of NADH is greater than that of succinate, high energy intermediates of oxidative phosphorylation are utilized to complete this reaction. The metabolic

[2] The relative amount of background and the NADH signals is highly dependent on the optical arrangement and the quality and transmission characteristics of the primary and secondary filters. The values given in Table I and II are from experiments using a 365 ± 15 mμ primary filter and a Wratten 2A gelatin filter cutting off all wavelengths below 405 mμ. Some experiments were performed using an interference filter with a block-shaped transmission window from 435–495 mμ, i.e., straddling the 465 NADH fluorescence peak. This provides a higher NADH contribution to the total and therefore percentage-wise, larger deflections (see Fig. 10). Except in some unusually favorable preparations the decrease of the total signal, however, necessitates the use of higher amplification resulting in more noise and, therefore, little or no advantage over the 2A filter.

significance of this fraction is not clear, but it is probably related to reductive biosynthetic reactions rather than to oxidative phosphorylation.

The toad muscle mitochondria respond to succinate (20 mM) with an increase in fluorescence equal to about one-third of the total state 5-2 signal. Addition of α-glycerophosphate (20 mM), however, produces an increase of 1-2%. The possibility exists, therefore, of a reduction of mitochondrial NAD by α-glycerophosphate arising from glycolytically formed dihydroxyacetonephosphate (Sacktor and Cochran, 1957). However, in the intact tissue the effect must be negligible on a quantitative basis.

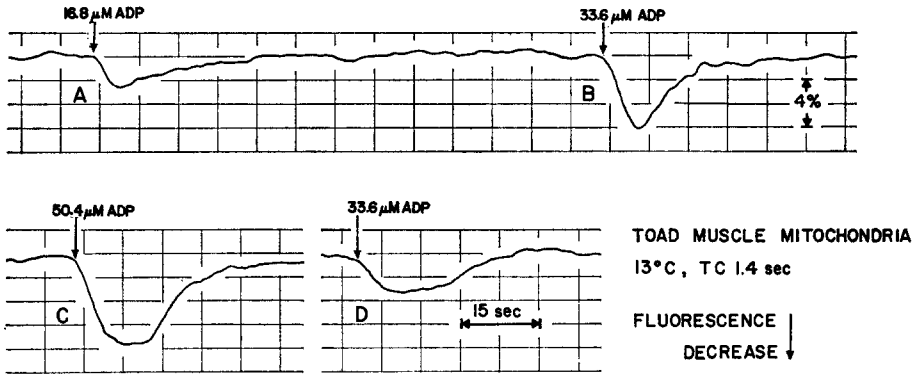

FIGURE 1. Fluorescence cycles of skeletal muscle mitochondria; effect of ADP additions on the fluorescence of mitochondria in state 4. A, B, C, without $MgCl_2$; D the same aliquot after the addition of 0.3 mM $MgCl_2$ resulting in 0.1 mM Mg^{++} and 0.2 mM Mg-EDTA complex. Incubation conditions, reaction medium + 2 mg/ml albumin + 10 mM Na-pyruvate + 2 mM malate. TC = time constant.

THE Δ FLUORESCENCE CYCLE OF MITOCHONDRIA DUE TO APD ADDITION

From previous spectrophotometric work with intact muscles, it was expected that the addition of ADP to an aliquot of suspended mitochondria in the resting state (state 4) would correlate with the transition from resting to recovery metabolism in the intact muscle. Special emphasis was, therefore, placed on the study of the state 4 to state 3 transitions and vice versa in the isolated mitochondria.

When ADP is added to suspended mitochondria in the resting state (state 4), an immediate decrease in fluorescence is observed. After a variable interval, the length of which depends on the amount of ADP added, the trace returns to the original state 4 base line. Fig. 1 shows several cycles of fluorescence changes induced by the addition of different amounts of ADP to toad muscle mitochondria in the presence of 10 mM pyruvate plus 2 mM malate and an excess of P_i (22 mM). A large number of similar cycles can be obtained from the same aliquot of mitochondria so long as adequate levels of oxygen

are maintained. Two parameters were chosen as quantitative measures of the intensity and the extent of the fluorescence changes: the peak height of the response (P_{max}) and the total area under the curve ($\int\Delta Fl$, time integral of the fluorescence cycle).

The relation between the concentration of the added ADP and the magnitude of P_{max} is depicted in Fig. 2 (upper left curve). The effect of increasing ADP concentrations conforms quite well to the shape of a saturation curve. This aspect is shown in Fig. 2 (right), in which the data are rendered in the manner of Lineweaver and Burk by plotting the reciprocal of the P_{max} as a

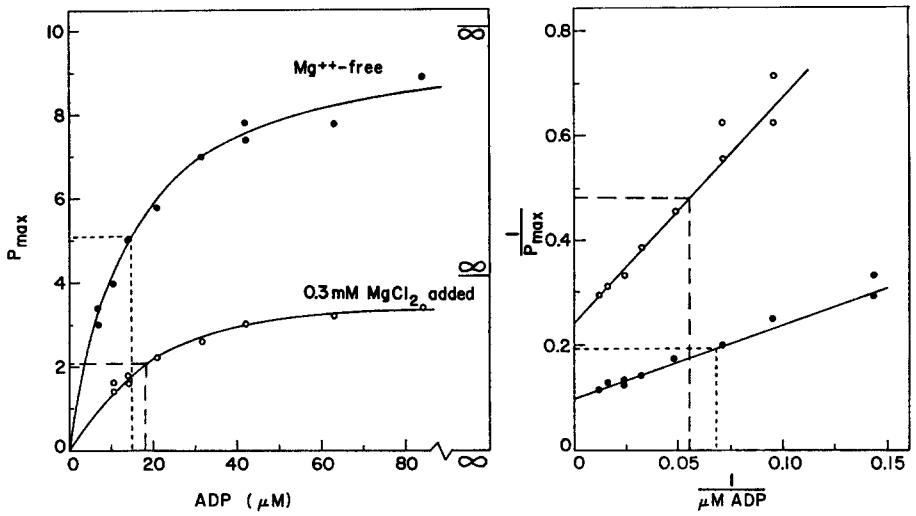

FIGURE 2. ADP saturation of the fluorescence response. Left, the peak of the fluorescence cycle expressed as per cent of the state 4 signal. Right, presentation of the same data in a double reciprocal plot. The solid line curves in the left-hand figure were calculated from the straight lines in the right-hand plots. The broken and dotted lines indicate the K_M values. The abscissae for the experiment with Mg^{++} refer to the added ADP concentrations; i.e., above the steady-state level.

function of the reciprocal of the ADP concentration. The intercept of the straight line of best fit with the y axis yields the extent of the P_{max} at infinite ADP concentration. This level is indicated by the short bar on the right-hand ordinate in Fig. 2 (left). The ADP concentration for half-maximal P_{max} (the apparent Michaelis constant, K_M) was calculated from Fig. 2 (right) as is shown by the dashed lines. The K_M is useful in the determination of the sensitivity of the system for ADP and thus for the calculation of the increases in internal ADP concentrations after contractions. A total of six such curves from four preparations (without added $MgCl_2$) yielded an average of 15×10^{-6} M ADP ($\pm$ 5 $\times$ 10^{-6}) for the K_M of the system at 12°C ($\pm$ 1°) whereas seven runs on five preparations yielded 26×10^{-6} M ($\pm$ 9 $\times$ 10^{-6}) at 24°C ($\pm$ 1°).

It was found that most of the preparations of muscle mitochondria were contaminated with a Mg^{++}-activated ATPase, probably of myofibrillar origin. The exclusion of Mg^{++} from the incubation medium successfully suppressed this ATPase activity. The occurrence of such contaminations can be used as a method for the further investigation of the interrelation between P_{max} (or the $\int\Delta Fl$) and the ADP concentration. The addition of $MgCl_2$ lessens respiratory control by increasing the hydrolysis of the ATP formed by oxidative phosphorylation and thus raises the ADP level. By judicious control of the $MgCl_2$ concentration it is possible to elicit higher rates of oxygen uptake accompanied by decreases in the fluorescence signal.

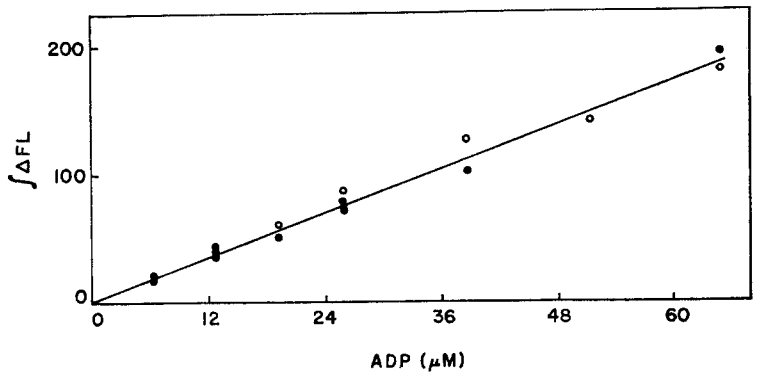

FIGURE 3. The area under the fluorescence response cycle as a function of ADP. Toad skeletal muscle mitochondria at 12.5°C. Abscissa, ADP concentration immediately after the addition; ordinate, planimeter readings in arbitrary units. Solid circles, reactions in the usual suspension medium; open circles, in the presence of 0.1 mM Mg^{++}.

The lower left-hand curve in Fig. 2 shows for the same preparation the relation between the P_{max} and the ADP concentration in the presence of 0.1 mM Mg^{++}. This amount produced a steady-state level of oxidation of NADH equal to approximately 50% of the P_{max} at infinite ADP concentration. Thus a steady-state concentration of about 10×10^{-6} M ADP must have been present in the second part of this experiment. Addition of ADP to this level produced relatively smaller but longer lasting cycles. The P_{max} values of these are plotted in Fig. 2. The K_M for the additional ADP in the presence of Mg^{++} was 18 μM. This difference is probably insignificant.

The area subtended by the trace from the moment of ADP addition until the base line has been reattained varies linearly with the amount of ADP added (Fig. 3). This parameter is the (time) integral of the fluorescence cycle; $\int\Delta Fl$ will be used as an abbreviation. On the recording charts it is measured most conveniently with a polar planimeter. In all experiments on toad muscle mitochondria as well as in more numerous ones on rat kidney mitochondria the linear relation shown in Fig. 3 was borne out. In this and other experi-

ments ADP additions were made under different initial steady-state levels induced by varying Mg^{++} levels. It is of some importance that the linear relation between $\int\Delta Fl$ and the ADP concentration is identical with and without Mg^{++}. This is qualitatively evident since in the presence of Mg^{++} the $\int\Delta Fl$ cycles although flatter, are prolonged (Fig. 1). The linear relation holds regardless of the basal oxidative rate and the initial steady-state level of oxidation-reduction of NADH so long as the substrate supply and the temperature are constant. Provided these conditions are met, the $\int\Delta Fl$ can be used for the comparison of different amounts of contractile activity, despite changes in the steady-state level of NADH due to changes in the resting rate of oxidative metabolism.

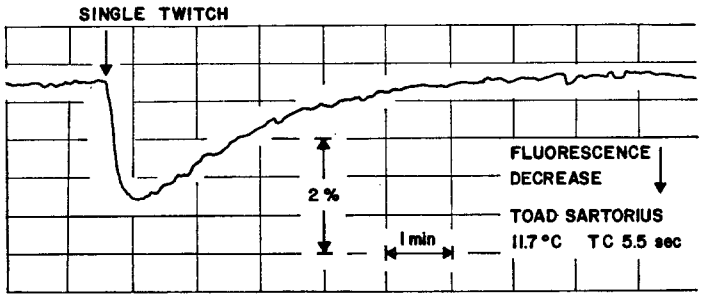

FIGURE 4. Fluorescence cycle induced by one isometric twitch. The muscle was equilibrated overnight at 10°C in Ringer's solution + 10 mM Na-pyruvate. Initial tension 4 g; length 105% of body length.

II. *The Fluorescence of Excised Muscles*

QUALITATIVE EFFECTS OF CONTRACTILITY ON THE FLUORESCENCE

In the presence of substrate and oxygen, contractile activity always results in a decrease in fluorescence. Fig. 4 shows a typical record of fluorescence decrease induced by a single isometric twitch at 12°C. At higher temperatures the peak (P_{max}) of such cycles is practically the same but the return to the base line is more rapid. Below 12°C the P_{max} tends to decrease slightly whereas the return is greatly protracted.

In a number of experiments aimed at the elucidation of the time relations of the fluorescence response, the time constant of the fluorometer was reduced to 1.4 sec and the recorder was run at a higher speed. The onset of the cycle has a time constant (time taken for the attainment of 0.632 of the final level) of approximately 6 or 7 sec, as shown in Fig. 5. Thus the utilization of an instrumental time constant up to this figure is warranted without danger of misrepresentation due to overdamping of the instrument. Although the records of such experiments were rather noisy, it was tentatively concluded that the onset of the fluorescence change occurs after the relaxation of the isometric tension

is complete (see Fig. 5). This is at variance with earlier results on the onset of cytochrome *b* oxidation during a single twitch observed spectrophotometrically which showed that the increased oxidation of cytochrome *b* commenced as relaxation began (Jöbsis, 1959). In the present study no further investigation of this difference was made.

A number of qualitative control experiments were performed to determine whether the decrease in fluorescence is caused by an oxidation of respiratory NADH. This was done under conditions where no change in NADH was to be expected after contractile activity; e.g., in the anoxic substrate-depleted states (states 5 and 2). In anoxia contractile activity does not elicit a fluorescence cycle[3] either with or without iodoacetate, although in the latter case small,

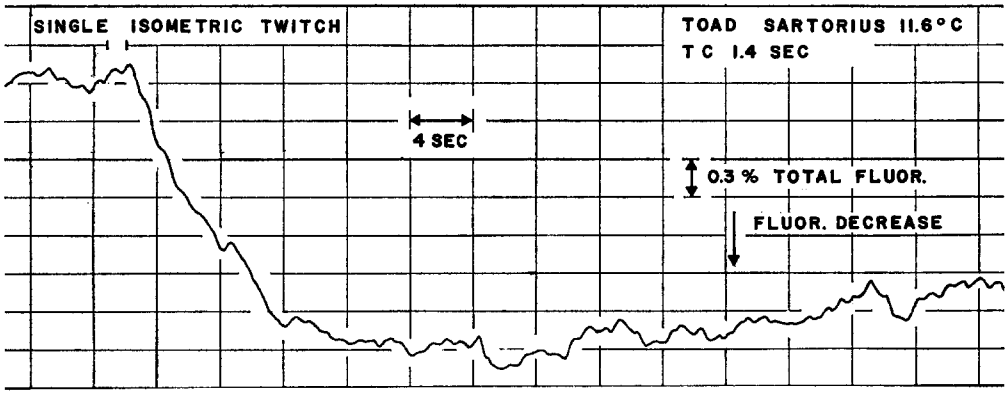

FIGURE 5. Initial kinetics of the fluorometric response to an isometric twitch. The short gap in the line just above the fluorescence trace indicates the duration of the entire twitch.

lasting increases do occur (see below). For the state 2 experiment, the substrate level was brought close to zero by repetitive twitching in the presence of 0.5 mM IAA and adequate O_2. Under these conditions a steady, low level of fluorescence is reached after which no fluorometric responses are observed following contractions. These qualitative controls, together with the wavelength characteristics of the fluorescence emission (Chance et al., 1962), provide good evidence that the observed fluorescence cycles are not indications of glycolytic activity, but rather of a respiratory function, most probably the oxidation of mitochondrial NADH.

When an anoxic muscle, not poisoned with iodoacetate, is stimulated to perform a number of contractions (10-30) at a rate of one per sec a small,

[3] In a small percentage of muscles contractile activity gives rise to a smaller and much more rapid, fluorometric effect, with a time constant of onset of approximately 2 sec and a return to the base line with a similar TC. Such deflections are considered to be motion artifacts. The occurrence of these responses has become less frequent with greater experience in the mounting of muscles in the holder.

lasting increase in fluorescence is observed. This is shown at the top of Fig. 6, where the changes observed are expressed as a percentage of the increase in fluorescence between the substrate-depleted and anoxic states (states 2 and 5). Each period of contractile activity induces an increase of only 0.5–1%. This amounts to 0.1–0.2% of the state 4 level, whereas a similar series of twitches with adequate oxygen would produce a response of 5–8%. The insignificance of the contribution of glycolytic NADH changes to fluorescence is also corroborated by the results shown in Fig. 8. In that experiment the absence or

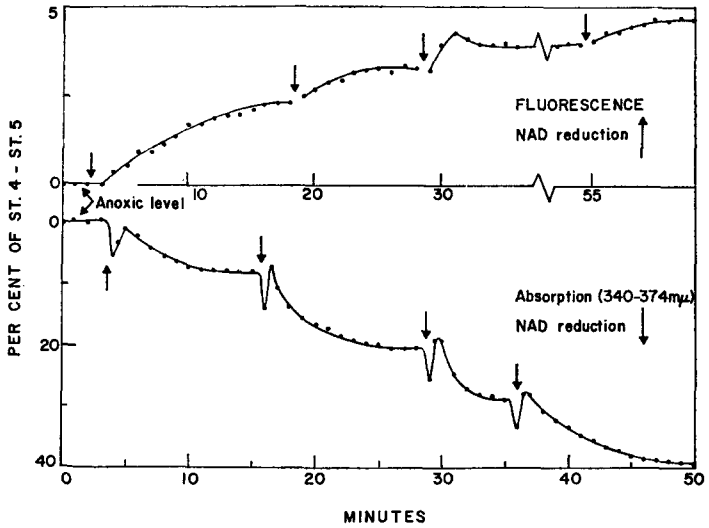

FIGURE 6. Comparison of fluorometric and spectrophotometric measurements of NADH changes in an anoxic muscle. The arrows indicate the times at which series of twitches were evoked at a rate of 1 cps. Each circle shows the average level of the trace over a 1 min period.

presence of IAA affects neither the $\int\Delta Fl$ nor the P_{max} of the fluorescence cycles elicited by single twitches. The lower graph of Fig. 6 shows a similar experiment in which NADH was measured spectrophotometrically by the double-beam method (measuring beam at 340 mμ). After a similar series of twitches a much greater increase in NAD reduction is noticed. The fluorometric effect is abolished by IAA. However, large changes in light scattering due to IAA prevent meaningful spectrophotometric observations on this point (see Jöbsis, 1963 *b*).

From the spectrophotometric observations it is concluded that under anoxic conditions a long lasting reduction of glycolytic NAD occurs after contractile activity. The fluorescence signal apparently contains a much smaller contribution from cytoplasmic NADH than from the mitochondrial fractions. This is in agreement with the 5- to 15-fold enhancement of NADH fluorescence in

isolated mitochondria over that of an equal amount of NADH dissolved in vitro (Estabrook, 1962; Avi-Dor et al., 1962). The above experiments show that the fluorescence of mitochondrial NADH is enhanced about tenfold in comparison with cytoplasmic NADH.

STEADY STATES IN THE INTACT MUSCLE

A number of experiments were conducted in an attempt to identify metabolic situations in the excised muscle which corresponded to the steady states in isolated mitochondria (see Table I). Eventually a procedure was found by which all five states could be induced in one muscle and these data are reported in Table II.

TABLE II

FLUORESCENCE LEVELS IN EXCISED TOAD SARTORII DURING VARIOUS METABOLIC STEADY STATES

Temperature, °C	12								24							
Condition	Fresh				Equilibrated				Fresh				Equilibrated			
State	1	3	4	5–2	1	3	4	5–2	1	3	4	5–2	1	3	4	5–2
	% of state 4			% of state 4				% of state 4				% of state 4				% of state 4
Fluorescence level	44	44	67	18	37	47	71	18	86	43	71	14	47	47	71	17
	37	38	64	17	39	40	70	21	62	40	69	13	50	49	71	19
	61	44	73	16	46	52	73	23	57	47	72	20	40	44	70	14
					59	51	71	17					53	44	72	16
					40	43	75	20								
Average	47	42	68	17	44	47	72	20	70	43	71	16	48	46	71	17

A typical experiment is shown in Fig. 7. On the left ordinate the intensity of fluorescence is expressed as a percentage of that in the resting state in the presence of 10 mM pyruvate (state 4); the right ordinate shows the state 5-state 2 difference as 100%. On the abscissa time proceeds from left to right as usual, but without strict proportionality. The dash at zero time indicates the fluorescence intensity of a muscle which was equilibrated for 16 hr at 10°C without substrate but with continuous bubbling of oxygen. In this type of experiment one to three twitches were always given before the first recorded point to insure proper positioning of the muscle in the light beam. The metabolic state at this point is perhaps best compared with state 1; i.e., resting metabolism in the presence of endogenous substrate. The difference between the fluorescence intensity in this and the reference state (resting + added substrate) is rather variable and no precise value can be assigned.

At the first arrow on Fig. 7 iodoacetate (0.5 mM) was added. Note that in this experiment a small change in fluorescence signal resulted. This seldom

occurs at 12°C but is almost always observed at 24°C, and is probably due to a higher resting metabolic rate which hastens the exhaustion of endogenous substrate.

Some 35 min after the IAA addition, several series of twitches were elicited which were accompanied and followed by a decrease in fluorescence until a lower, stable level was attained. At this point additional contractile activity did not result in further attenuation of the fluorescence signal. The level in

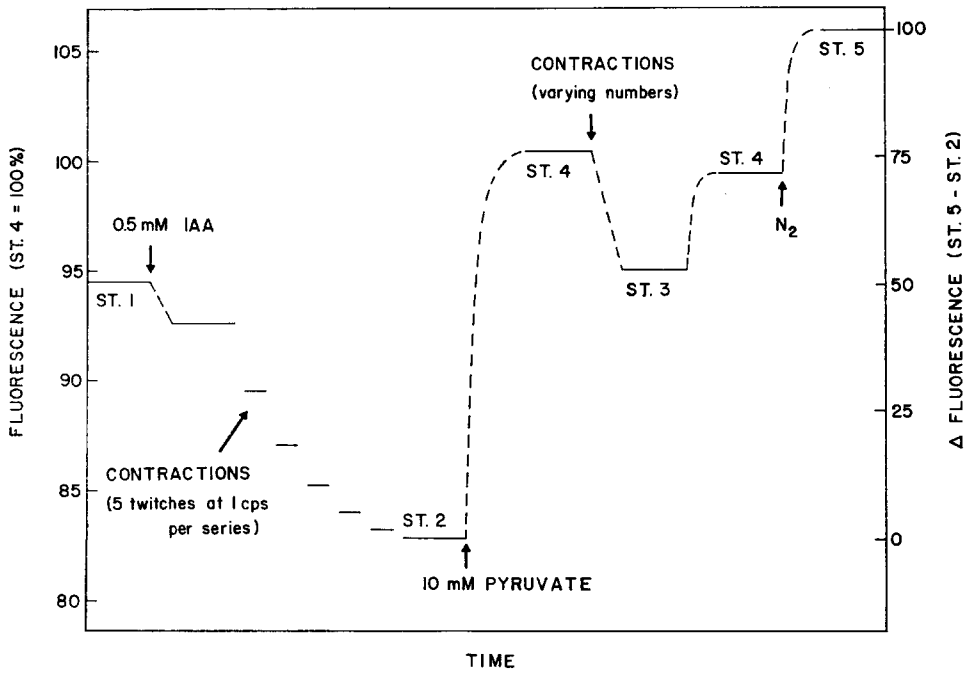

FIGURE 7. Assessment of the fluorescence signal in states 1 through 5. Toad sartorius, 12°C, aged overnight without added substrate. The ordinates are labeled in different units (see text). The abscissa shows the progress of time from left to right without strict proportionality. The entire experiment took approximately 4 hr.

this condition is relatively stable and is considered to be equivalent to state 2, the substrate-starved state. Little recovery towards the original level of fluorescence occurs during a period of 15-30 min, but a definite recovery can be measured in 2-3 hr at 12°C. The reducing equivalents for the oxidative recovery metabolism that appears to take place over this period are probably derived from amino acid and fatty acid metabolism. At 24°C a contracture usually develops before much evidence of this recovery is seen.

When sodium pyruvate (10 mM) is added to the solution bathing the sartorius (Ringer's solution + 0.5 mM IAA), a 10-15% increase in fluorescence is observed. The time course of this increase is in approximate agreement with

the expected time course of diffusion of the pyruvate from the solution into the muscle: the half-maximal effect is reached in about 8 min and the trace comes to a stable level in 30-35 min. The level thus attained is considered to be equivalent to state 4, the resting state. In this condition, oxidative metabolism is limited by the low ADP concentration and NAD reduction is high due to a sharply curtailed flow of reducing equivalents from substrate to oxygen. This is the state in which most experiments, aimed at the elucidation of the effect of contractility on fluorescence, have been performed. No further increase in fluorescence level can be obtained by increased levels of added pyruvate. At 12°C respiratory NADH appears to be saturated by 5 mM external pyruvate; at 24°C, however, 10 mM is required. These values agree well with those from similar experiments on cytochrome *b* and NAD performed with the spectrophotometric method (Jöbsis, 1963 *b*).

With a stable resting state, several series of twitches, of varying number but given at 1 cps, were elicited. The reciprocal of the P_{max} for each series of twitches was then plotted as a function of the reciprocal of the number of twitches in each series in the manner of Lineweaver and Burk. Extrapolation to an infinite number of twitches yielded the theoretically attainable maximal degree of oxidation of mitochondrial NADH in activity respiration; i.e., state 3. This procedure is more extensively presented in the next section (Figs. 13 and 14).

The maximum level of reduction of respiratory NAD was determined by the induction of anoxia. N_2 was bubbled through the solution until an increase in fluorescence signaled the beginning of anoxia, whereupon bubbling was stopped, and the muscle was allowed to attain a completely anoxic level in the unstirred solution. To accelerate this, a few twitches were elicited from time to time to increase the rate of oxygen consumption. Contractile activity, under completely anoxic conditions in the presence of IAA, induces no further changes in fluorescence, either in the steady-state level or, in most muscles, in the form of transients or "motion artefacts."

Addition of cyanide (3-6 mM, an excess of that required to inhibit the respiratory chain) induces no further change in fluorescence. In a number of muscles a contracture started to develop slowly at this point. This was commonly associated with a slight decrease in fluorescence signal. The well known increase in opacity which takes place under such conditions may underlie this effect.

In Table II the levels of the steady state derived from the above experiments are presented in a manner similar to that in Table I. Instead of listing the data as percentages of the total fluorescence in state 4, they have been calculated on the basis of a 100% value assigned to the difference in fluorescence between the substrate-starved state (state 2) and the anoxic state (state 5). This was done to make them more directly comparable with the data of Table

I. Good conformity to the fluorometric data from isolated mitochondria is exhibited. The only discrepancy appears to be the difference in fluorescence level between states 4 and 5. Whereas Chance and Williams (1955 *b*) found that NAD was maximally reduced in both conditions, the present data on isolated mitochondria and on the intact sartorii show respectively 10-15 and 25-30% less fluorescence in state 4 than in state 5. This is interpreted as signifying a less than maximal reduction of NAD in the resting state (state 4).

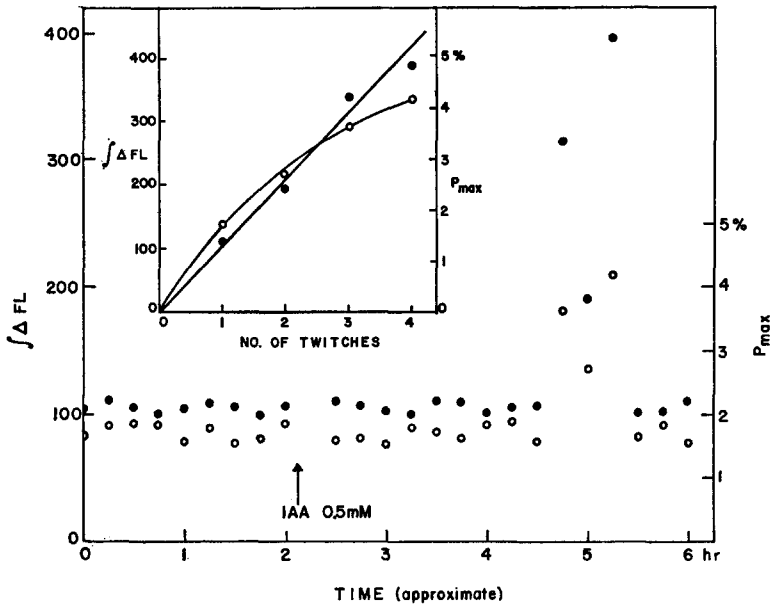

FIGURE 8. Reproducibility of twitch-induced fluorescence cycles in the absence and in the presence of IAA. Single twitches were elicited about every 12–15 min. Towards the end of the experiment some activity of two, three, and four twitches was given at 1 cps. In the inset the relation to the number of twitches is shown. Filled circles, $\int\Delta$Fl; open circles, P_{max}. Toad sartorius, 12.5°C, aged overnight with added pyruvate.

III. *Muscular Contraction and the Δ Fluorescence Cycle*

From the first successful experiments it was clear that greater contractile activity gives rise to larger Δ fluorescence cycles. For the remainder of this article various correlations will be explored, specifically the dependence of the P_{max} and the $\int\Delta$Fl on the number of and the interval between single twitches.

Fig. 8 shows the results of an experiment designed to illustrate the variability of P_{max} and $\int\Delta$Fl and the lack of an effect of IAA on the fluorescence cycles elicited by single twitches. The abscissa again is a time scale without strict proportionality to actual time. However, the single twitches were given at a more or less standard rate of 1 every 12-15 min. Both the P_{max} and the

$\int\Delta Fl$ are quite constant, regardless of the addition of IAA. The variability of the data for the P_{max} is approximately $\pm$ 7% and that of the $\int\Delta Fl$ is about $\pm$ 5%. Towards the end of the experiment a few series of twitches were given at a rate of 1 per sec. The result of this part of the experiment is shown in the inset of Fig. 8 where the P_{max} and the $\int\Delta Fl$ are presented as a function of the number of twitches.

The peak height (P_{max}) of the fluorometric response increases with the number of twitches. This is shown in Fig. 9 for a short sequence of cycles. With increasing numbers of twitches per series the value of the P_{max} increases, while the shape of the curve changes in two aspects: (*a*) the simple peak and monotonic decline are replaced by apeak-and-plateau type of response (see also Fig. 10), and (*b*) the trace tends to overshoot the base line and to return only after 5 to 10 min (see Figs. 9 and 10). Although these two changes appear

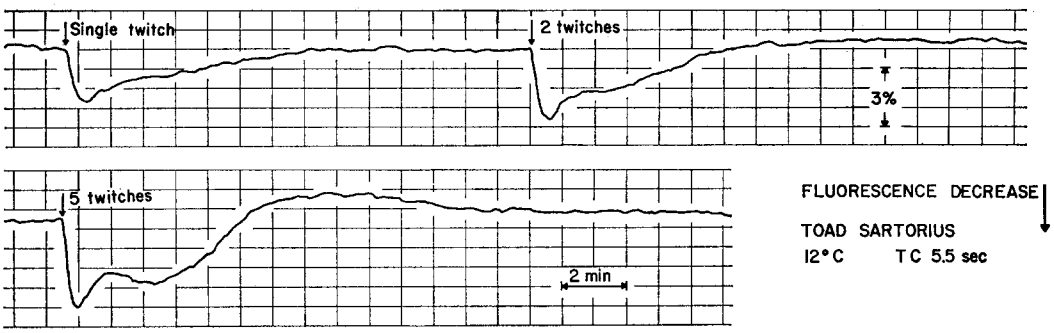

FIGURE 9. Fluorescence cycles elicited by different amounts of contractile activity. The twitches occurred at a rate of 1 cps. The bottom and top strips are a continuous record.

after about the same number of twitches per series they are probably not caused by the same process since addition of IAA does not eliminate the occurrence of the plateau but does prevent the overshooting of the base line. This holds true for several hours after the addition of IAA. Shortly before the usual contracture occurs however, the cycles lose their diphasic aspect; i.e. the response to 15 or 20 twitches differs only in size from that of a one twitch cycle. Since the moment of appearance of a contracture is highly variable between preparations (2-8 hr), it appears that there is some chance of a significant relationship between the two occurrences. From these observations it is deduced that the overshoot beyond the base line is related to glycolytic activity whereas the plateau is not.

From a qualitative point of view it would appear that the increased fluorescence of the overshoot might be caused by the fluorescence from the glycolytic reduction of cytoplasmic NAD. From quantitative considerations, however,

this explanation is untenable. If the small increases in fluorescence observed after contractions during anoxia (Fig. 6) are, in fact, reflections of glycolytic NAD reductions, then this contribution is approximately 0.025% of the state 4 signal for each twitch (or about 0.1% of the states 5-2 fraction). When an overshoot occurs the total amount of the effect is not merely equal to the overshoot value but should be calculated from the decreased fluorescence level which would have been maintained in its absence. In the last record of

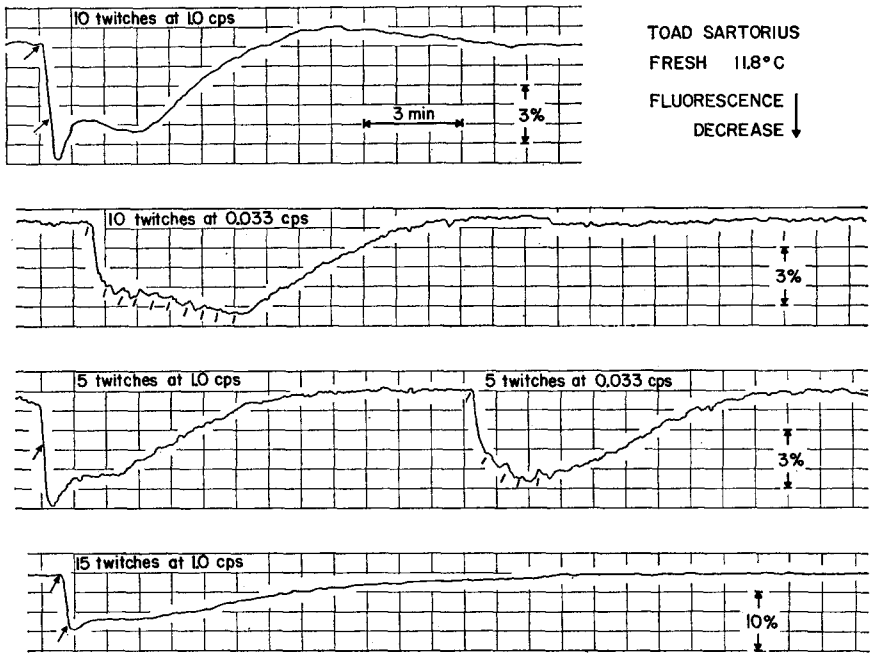

FIGURE 10. Effect of twitch rate on the fluorescence cycles in the absence (top two records) and in the presence of 0.5 mM IAA (last three records). Note the change in sensitivity for the last record. At slow twitch rates each contraction is indicated by a dash. At higher rates the total period of stimulation is shown by the two arrows. The IAA was added approximately 40 min before the third record.

Fig. 9 for example, the attenuation of the fluorescence cycle amounts to approximately 5% of the state 4 level or about 1% per twitch. The rapid attenuation of the cycle and the subsequent overshoot can, therefore, not be attributed to the direct contribution of glycolytic NADH to the total signal.

The theoretical possibility also exists that glycolysis effects a more direct reduction of mitochondrial NADH. This hinges upon the fact that certain substrates (succinate, α-glycerophosphate) produce a reduction of a mitochondrial NAD fraction (NAD_2) not directly involved in oxidative phosphorylation (Chance and Hollunger, 1961). Since dihydroxyacetonephosphate

arising from glycolysis can be reduced to α-glycerophosphate (Sacktor and Cochran, 1957) the above mentioned reduction of mitochondrial NAD becomes a definite possibility after vigorous contractile activity. In view of the very small effect noted with isolated mitochondria, the significance of this pathway is probably small if it is indeed present at all.

In a limited number of preparations the overshoot is followed by several oscillations in the fluorescence. This occurs only after a relatively large number of twitches in rapid succession. Such a record is presented in Fig. 11 on a condensed time scale. These observations are highly suggestive of the recently

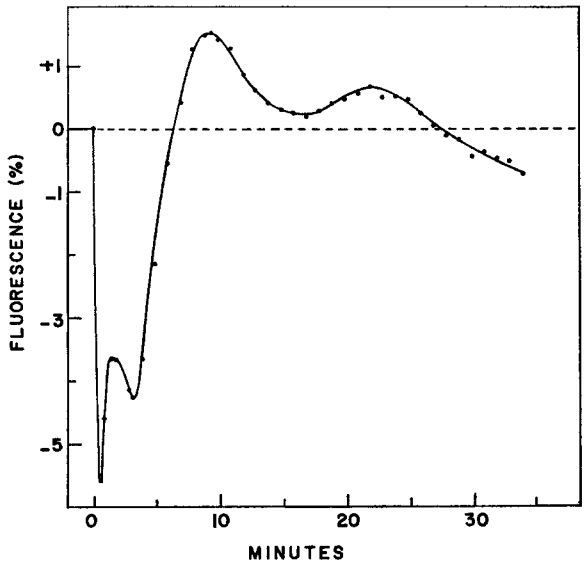

FIGURE 11. Oscillatory fluorescence changes in response to 15 twitches at one per sec. Toad sartorius, 12°C. Equilibrated overnight in Ringer's solution + 10 mM pyruvate at 10°C.

described oscillations in the fluorescence signal from yeast when glycolysis is markedly stimulated, most commonly upon the transition from aerobic to anaerobic metabolism (Hommes and Schuurmans Stekhoven, 1964), and it has been shown that in yeast, oscillations in the reduction level of cytoplasmic NAD underlie this phenomenon (Ghosh and Chance, 1964). Under the present conditions anoxia does not occur, as has been shown by spectrophotometric observations (Jöbsis, 1963 *b*). In that same study it was proven, however, that aerobic glycolysis can be greatly stimulated by contractile activity. In view of the negligible contribution of glycolytic NADH to the fluorescence it must be concluded that the oscillatory activity of the glycolytic system is reflected in the reduction level of respiratory NADH.

This conclusion gains strength from the observations of Betz and Chance (1965 *a*, *b*) who have demonstrated that oscillations in the NADH level in yeast cells are correlated with fluctuations in the ADP concentration. The present oscillations in the reduction level of respiratory chain NADH could, therefore, constitute secondary effects caused by fluctuating ADP levels due to glycolytic oscillations. It is probable that even the simpler overshooting of the base line constitutes the initial phase of an abortive oscillation. In that case the temporarily increased reduction level of NADH would reflect a lowered ADP level rather than an increased substrate concentration as was concluded before (Jöbsis, 1963 *b*).

With an increase in the interval between individual twitches to more than 5 sec a change in the shape of the response cycle starts to occur. At 12°C and an interval of 30 sec subsequent twitches coincide approximately with the peak of the fluorometric response to the previous twitch. From the second trace of Fig. 10 it is seen that after the last contraction a rapid monotonic return to the base line commences. A plateau is not evident. In this case, therefore, both the plateau and the base line overshoot have been eliminated by increasing the interval between twitches. Apparently, therefore, it is not merely the amount of ADP released but the amount per unit time that underlies the oscillatory behavior and also determines the appearance of the plateau. It is thought that the glycolytic system is responsible for the overshoots and the oscillations of the fluorometric trace. Since IAA does not abolish the peak-and-plateau type of response, it is suspected that the buffering of the ADP increase by the ~P stores may only be complete after the mitochondria have signaled the temporary presence of a much higher ADP concentration.

The experiments presented above were performed mainly in the winter and spring. When repeated in the summer the results were essentially the same except for a lack of base line overshoots after high rates of activity at 10-12°C. However, at 24°C they were still present. In all seasons incidental fluorometric observations showed that temperature has a greater influence on glycolysis than on electron transport. Evidence of glycolytic activity such as base line overshoots is progressively less easily elicited with decreasing temperatures. The fluorometric responses of respiratory chain NADH, however, remain relatively constant or may even show a slight increase in the cold. This is in agreement with the fact that the Q_{10} of glycolysis in frog muscles is almost 4 between 5 and 15°C, whereas the oxidative metabolism measured as the oxygen uptake rate responds to this 10°C temperature rise with a threefold increase (Meyerhof, 1930). Both figures pertain to experiments done under conditions closely approximating state 3 conditions. The seasonal effect on the occurrence of overshoot could be explained by postulating that an acclimatization had taken place so that in summer the same interrelations be-

tween the two enzyme systems were present, say, at 24° as at 10-12°C in the winter.

P_{MAX} AND THE NUMBER AND FREQUENCY OF TWITCHES

The quantitative correlation between the number of twitches and the P_{max} was studied extensively. In Fig. 12 the results of a typical experiment are shown in which besides varying the number of twitches per series, the rate of twitching was also varied between 1 and 0.033 cps. The two curves express the results already implicit in Figs. 9 and 10. On the right-hand side of Fig. 12 the data are presented in the reciprocal Lineweaver-Burke plot. Straight lines

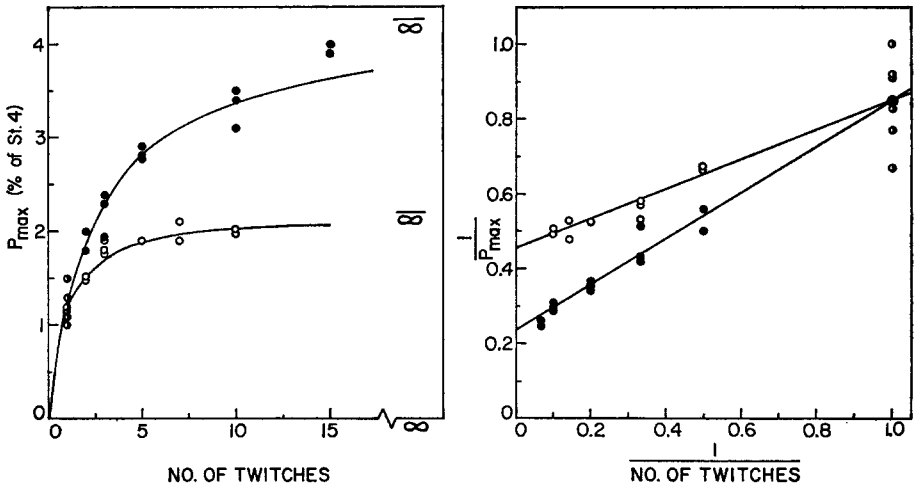

FIGURE 12. Saturation of the fluorescence response to twitching at two rates. Open circles, rate of 0.033 cps; filled circles, 1 cps. The curves drawn in the left-hand graph were calculated from the lines of best fit in the right-hand one. The dot and concentric circle in the right-hand graph represent the average of all single twitch runs. Toad sartorius, 11.5°C, equilibrated overnight.

were fitted through the two sets of data. The intercepts with the y axis were used to calculate the P_{max} for an infinite number of twitches. These levels are given by the short bars at the right-hand side of the left graph of Fig. 12. From here on they will be referred to as $P_{max\infty}$.

From this and similar experiments it is clear that the saturation of the oxidative phosphorylation system is highly dependent on the rate as well as on the number of twitches given within one series. Thus the determination of the state 3 (activity respiration) level of fluorescence is not absolutely straightforward. Transphosphorylating systems tend to lower the change in ADP concentration produced by contractile activity. Only comparison of the effect of different rates on the $P_{max\infty}$ level can resolve the question of the ultimate level of fluorescence in state 3; i.e., in the presence of an excess of ADP. This

point has been explored in several experiments two of which are presented in Fig. 13. Here the $P_{max\infty}$ has been plotted as a function of the frequency of twitching. Clearly at the higher rates a trend occurs toward saturation at a fluorescence level 5% below that of state 4. The main decrease in the extrapolated $P_{max\infty}$ level starts at frequencies below 0.3 cps. Apparently a significant amount of buffering of the ADP released by each twitch can take place between twitches if the interval is about 3 sec or longer. This is also approximately the rate at which the peak-and-plateau type of response is less pronounced, but this point is not easily ascertained in the traces and the last generalization must remain tentative. It is clear, however, from Fig. 13 that

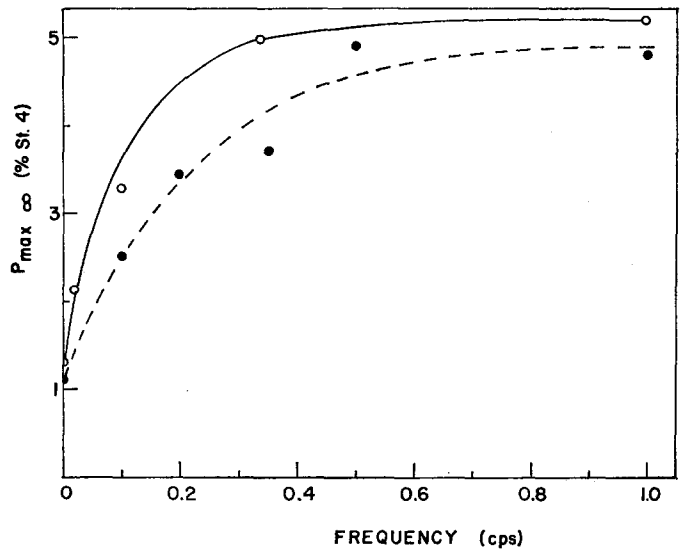

FIGURE 13. Saturation of the $P_{max\infty}$ at different rates of twitching. Two toad sartorii, 12°C, equilibrated overnight with pyruvate.

at a rate of 1 cps a plateau level has been established for the $P_{max\infty}$, and therefore, by extrapolation of the P_{max} to an infinite number of twitches at a rapid rate, the state 3 level of NAD reduction may be determined. The state 3 levels reported in Table II were determined in this fashion.

MUSCULAR ACTIVITY AND $\int\Delta FL$

For the measurement of the area under the fluorescence cycles a number of conventions concerning base lines needed to be adopted. Two types of problems were encountered, (*a*) the permanent establishment of a new fluorescence level at the end of the cycle and (*b*) overshoots or oscillations. In cases of permanent discrepancies between base lines before and after a cycle the following rule was observed. If the discrepancy amounted to more than 20% of the

P_{max} of the cycle, the run was discarded. When smaller than 20% the final base line was used and extrapolated horizontally back along the time axis until it met either the downstroke of the onset of the fluorescence change or the vertical, upward extrapolation of the beginning of the fluorescence response. This practice implies that such base line shifts are attributable to sudden changes occurring during the contractile activity which might mean a permanent though subtle shift of the alignment of the muscle in the light beam. This was tested in an activity series comprising one to four twitches during which some cycles showed such base line discrepancies and others did not. Little additional variability was found between the two types of results.

When oscillations occur the $\int\Delta Fl$ can no longer be meaningfully correlated with the total contractile activity. The trace returns to and overshoots the base line more rapidly with increasing numbers of twitches and a straight, horizontal base line is patently inapplicable. Similarly it is not warranted to draw a line to the peak of the first oscillation. Under these conditions the data are not amenable to analysis utilizing the $\int\Delta Fl$. This is emphasized by the lowest curve of Fig. 14 as discussed below.

The relation between the number of twitches and the $\int\Delta Fl$ was explored extensively and Fig. 14 presents the most complete experiment in which both rates and number of twitches were varied in the absence and in the presence of IAA. On the abscissa the sum total of the peak isometric tension produced has been used rather than the number of twitches. This parameter will be referred to as the cumulative, isometric force, ΣF_0. In the presence of IAA the twitch tension has a tendency to decline, especially for large numbers delivered at a rapid rate. Thus starting from the origin and proceeding to the right the clusters of points represent series of 1, 2, 3, and 4 twitches, etc. with tensions of approximately 60 g per twitch.

The relation between total cumulative tension and $\int\Delta Fl$ is linear for at least three twitches independent of the rate and the presence of IAA. This is commonly the case at 10-12°C whereas at 24°C the linear range is more limited. In the absence of IAA at a rate of 1.0 cps the fluorescence cycles become relatively too small at more than three twitches per series. This is due to the tendency toward a more rapid return towards and beyond the base line. At approximately five twitches per series at this rapid rate base line overshoots start to occur. For the remainder of the lowest curve of Fig. 14 the $\int\Delta Fl$ was calculated from the extrapolation of the initial base line. The very rapid turnoff of the straight line relation between ΣF_0 and $\int\Delta Fl$ testifies to the inadequacy of the $\int\Delta Fl$ as a useful measure of recovery metabolism when oscillatory overshoots occur. At a rate of one twitch every 30 sec overshooting of the base line is seldom seen and, therefore, the study of the $\int\Delta Fl$ can be meaningful. It is observed that the linear range of the relation between the $\int\Delta Fl$ and the cumu-

lative force persists until approximately five twitches. With larger numbers of twitches the linear relationship is lost.

In the presence of iodoacetate the linear range between ΣF_0 and $\int\Delta Fl$ extends to approximately seven twitches and the difference between 1.0 and 0.033 cps has disappeared (Fig. 14, top curve). From this it is concluded that the earlier and more extensive loss of the linear relation in the absence of IAA is due to glycolytic activity. The loss of the linear relation between the ΣF_0 and the $\int\Delta Fl$ in the presence of IAA may indicate a loss of phosphate acceptor

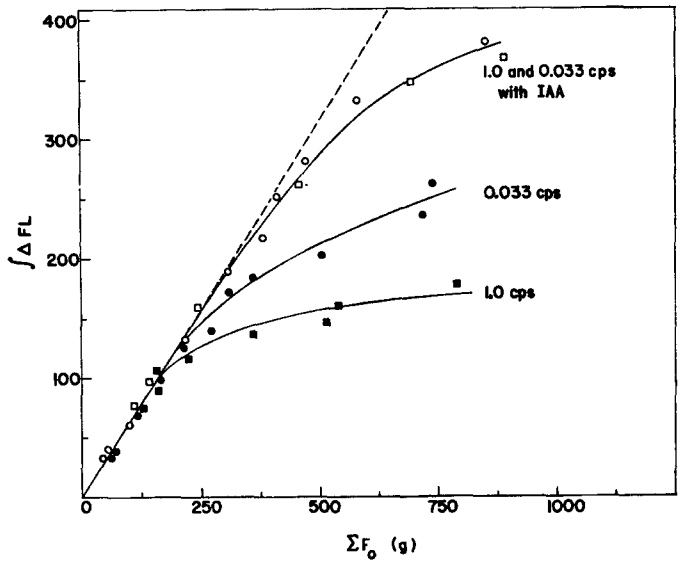

FIGURE 14. The relation between the total isometric tension in series of twitches and the integral of the fluorescence cycle. Effect of frequency of twitching before and after treatment with iodoacetate. Toad sartorius, 11°C, equilibrated overnight in Ringer's solution with 10 mM pyruvate.

due to catabolism of AMP, formed when two ADP molecules undergo mutual transphosphorylation by the action of myokinase, thereby yielding one ATP and one AMP (= adenylic acid). A fraction of the latter will then be converted by adenylic deaminase to inosine. Since this last reaction is relatively irreversible, the inosine is further catabolized and this AMP fraction is, therefore, lost. In fact both ADP molecules do not show up in the balance sheet of oxidative phosphorylation, since one was converted to ATP by the myokinase reaction.

The above experiment is fairly typical for muscles from winter toads at 12°C. In summer the linear range in the absence of IAA tends to be slightly greater at the lower temperatures and the 1.0 and 0.033 cps curves are less discrepant.

In conclusion it can be stated that after more than three to four twitches at 12°C glycolytic activity starts to become functionally significant in aerobic recovery metabolism. From the difference between the extrapolated straight line and the curves found in the absence of IAA it appears that after 10-15 twitches recovery via oxidative phosphorylation accounts for less than half the total.

DISCUSSION

One of the two major aims of this study was to identify that fraction of the fluorescence elicited by 365 ± 30 mμ excitation light that can be ascribed to the NADH associated with the oxidative phosphorylation reaction of the respiratory chain. The other major goal was the subsequent investigation of the possibility of utilizing the signal from the respiratory NADH fraction as a quantitative index to the recovery metabolism. These two aims will be further discussed below in the sequence given above.

First, however, the most eagerly awaited result was the extent of possible motion artefacts. In the earliest spectrophotometric work, muscles were constrained by light compression between Lucite plates, perforated to allow for the diffusion of O_2 (Chance and Connelly, 1957). Subsequently it was found that a very slight stretching of the muscle over a convex surface was sufficient to eliminate fluttering in the circulating Ringer's solution and to insure repositioning after a contraction. This system was, therefore, adequate for steady-state measurements; for instance, before and after contractile activity (Jöbsis, 1963 *a* and *b*). For spectrophotometry during and immediately after a contraction a greater amount of stretch and a higher degree of curvature of the convex surface were needed (Jöbsis, 1959, 1963 *b*).[4]

In the present study the sartorius was without constraint along its entire length; only the two ends were fixed. In all experiments the initial tension was 4 g, just sufficient to keep these rather large (300 - 400 mg) muscles at approximate body length. For the elimination of all motion except in the longitudinal direction, the clamp across the tendon at the origin is greatly superior to the usual mounting with the acetabula as anchor points. With well mounted muscles a contraction does not result in a deflection of the trace under conditions in which no change in the respiratory NADH is expected (states 2 and 5). Another case in point is the record shown in Fig. 5 where a twitch (lasting 1 sec) did not produce a significant deflection in the fluorometric signal of the

[4] In addition it was learned that for such measurements it is essential to minimize the distance between the sample and the light-sensitive cathode of the photomultiplier tube. This increases the solid angle subtended by the device and thereby eliminates to a great degree the light losses stemming from changes in the degree of diffraction and scattering. This of necessity follows directly from the observation of Sleator (1953), whose invaluable advice and generous assistance were inadvertently not acknowledged at the time of the previous publication (Jöbsis, 1963 *b*).

resting state until well after the completion of relaxation. Here the recording time constant (1.4 sec) was sufficiently rapid to have shown a response to an artefact.

At first sight this absence of a significant effect of contractile activity on the fluorescence is unexpected in view of the well documented changes in the characteristics of light transmission which take place (Sleator, 1953; for further references see Jöbsis, 1963 *b*). The essential difference in the optics is that the fluorescing area of the muscle, rather than being merely a transmitting medium, constitutes a light source. Although the intensity of the fluorescence is recorded from the illuminated surface the light emitted by the fluorescing molecules must nevertheless traverse some distance through the tissue. The contribution to the total signal by layers progressively further away from the illuminated surface decreases logarithmically. The space constant of this decrement is related to wavelength-nonspecific absorption, scattering, diffraction, and other losses rather than to the absorption by NADH which is minute. Studies by Mauriello and Ramirez showed that in frog sartorii more than 90% of the signal is derived from the first millimeter, 50% of which is contributed by the first 0.2-0.3 mm (personal communication). Thus the contribution of the deeper layers is sizable.

The absence of light-scattering artefacts is probably due to the fact that each fluorescing molecule can be considered a point source. Since the average direction of the mission is random, interposition of a scattering medium does not materially alter the situation and neither does a change in the scattering of the medium during contraction. Certain, hypothetical light quanta which would have reached the detector from the relaxed muscle but are now lost, will be compensated for by quanta which originally would not have reached the detector. Therefore, contraction artefacts would only result from a change in either the distance between sample and detector, or in the optical density (absorption) of the sample. The first can be avoided by proper mounting, the second apparently does not occur to any appreciable extent.

The Contribution of Respiratory NADH to the Fluorometric Signal

In earlier studies it was established that NADH contributes significantly to the fluorescence elicited by 365 mμ light from yeast and bacteria (Duysens and Amesz, 1957), from isolated mitochondria (Chance and Baltscheffsky, 1958), and from intact tissues (Chance and Jöbsis, 1959). This conclusion was based partly on the wavelength characteristics of the excitation and emission spectra (Avi-Dor et al., 1962; Chance et al., 1962), partly on specific inhibitor studies (Chance et al., 1962), and partly on the quantitative correlation of the change in fluorescence intensity with analytically determined changes in the NADH level of the sample (Williamson, 1965). Further confirmation came from the highly elegant studies of Lübbers et al. (1965) on the synchrony of the kinetics

of the fluorescence changes with those of the reduction of cytochromes during and after an anoxic interlude. However, from these studies it also became clear that a considerable amount and sometimes even the major part of this fluorescence are not derived from the NADH associated with the respiratory chain. In fact, most of the signal is almost certainly not derived from any NADH fraction; this notwithstanding the fact that the emitted light exhibits a maximum around 450-470 mμ; i.e., in the same general region as NADH. Thereby it becomes clear that from purely spectral characteristics it is difficult if not impossible to identify the fluorescence at these wavelengths as derived from NADH; and even more so to pinpoint the fraction associated with oxidative phosphorylation.

The nature of the material(s) emitting the remainder of the observed fluorescence is quite unknown. From the great stability of the base line it appears, however, that its concentration does not fluctuate appreciably within the time limits of the present experiments. The contribution of this fraction to the total fluorescence varies from tissue to tissue. In the toad sartorius and in fact in most of the soft, vertebrate tissues studied thus far, the contribution of this kinetically inactive fraction is not overwhelming. It has become clear, however, that in each tissue the respiratory chain NADH may or does contribute a different portion to the total fluorescence emitted.

Contributions from NADPH and NADH not Associated with Oxidative Phosphorylation

Several distinct fractions of NAD and NADP exist in the cell, the major division being between intra- and extramitochondrial fractions. Only part of the intramitochondrial NAD is directly involved in the transfer of reducing equivalents from substrates to the flavoproteins of the respiratory chain and hence via the cytochromes to oxygen. This fraction is kinetically most actively involved in the process of oxidative phosphorylation and is designated $NADH_{(1)}$. Another mitochondrial fraction, $NADH_{(2)}$, is reduced indirectly from substrates such as succinate and α-glycerophosphate via a special flavoprotein fraction. The reaction is mediated by electron flow in the reverse direction; i.e., towards NAD^+. Since this constitutes a flow towards a negative redox potential, utilization of high-energy bonds accompanies this thermodynamically unfavorable process (Chance and Hollunger, 1961). The function of $NADH_{(2)}$ is not well known but it possibly serves a role in reductive, synthetic reactions. (For a detailed discussion, see Jöbsis, 1964 *a*.)

The fact that α-glycerophosphate formation is an adjunct of glycolytic activity may well account for a small part of the cutdown of the fluorescence cycle seen when glycolysis becomes activated. Experiments with toad skeletal muscle mitochondria have shown that $NADH_{(2)}$ can be partially reduced by this substrate. However, this amounts to only 1-2% of the total state 4 signal

when excess amounts of α-glycerophosphate are utilized. Although some effect is not precluded, it is probably not very important in these experiments.

Another NAD moiety functions in conjunction with the glycolytic chain. The enzymes constituting this pathway are freely dissolved in the fluid, intracellular phase and hence this complement of NAD is usually designated the "cytoplasmic" fraction. In view of the considerable activity of this pathway in the toad sartorius muscle (Jöbsis, 1963 *b*) some interference by this fraction with the measurement of the signal from respiratory chain NADH was expected. Fortunately, this problem did not materialize since the fluorescence of the mitochondrial NADH was found to be greatly enhanced compared to that of the cytoplasmic fraction (see Fig. 6). The enhancement of the quantum efficiency for NADH fluorescence when bound to enzymes has been described extensively (Velick, 1961). The above observations could be interpreted as showing that a larger percentage of the mitochondrial NADH is bound than is that of the cytoplasmic fraction. Conversely it might be that a greater degree of enhancement is responsible for the difference. Be this as it may, the relative enhancement of the signal derived from the mitochondrial fraction as compared with that of the cytoplasmic NADH associated with the glycolytic chain, eminently furthers the measurement of oxidative recovery after contractile activity.

Finally, interference with the fluorometric measurement of oxidative recovery metabolism could arise from fluctuations in the level of NADPH. However, in muscle tissue this coenzyme is present in much lower concentration than NAD. Together the intra- and extramitochondrial fractions amount to about 3% of the total concentration of nicotineamide coenzymes (Glock and McLean, 1955). It has also been shown that mitochondrial NADPH has a three to four times smaller fluorescence efficiency than mitochondrial NADH (Avi-Dor et al., 1962). Furthermore, when the kinetics of NAD and NADP reduction were determined by chemical analysis the redox kinetics of NADP were found to be much slower even on a time scale of minutes (Williamson, 1965; Chance, Schoener et al., 1965; Chance, Williamson et al., 1965). In these studies it was also shown that fluctuations in the fluorescence signal correlate well with changes in NADH concentration and vary poorly with those in the intracellular NADPH. These various measurements rule out the possibility of a significant interference of NADPH fluorescence with the utilization of $NADH_{(1)}$ fluorescence as an index to recovery metabolism.

In conclusion, it can be stated that interference from NADPH, nonmitochondrial NADH, and from mitochondrial $NADH_{(2)}$ has been ruled out on qualitative and partially quantitative grounds. The theoretical possibility exists, however, that some unidentified component of the respiratory chain gives rise to the fluorometric cycles. Until such an hypothetical compound has been identified and has been shown to duplicate the special characteristics

and the quantitative steady-state relation (following paragraphs) to which NADH should conform, the labile fraction of the fluorometric signal can with some confidence be assigned to the mitochondrial $NADH_{(1)}$ fraction.

The Steady-State Oxidation-Reduction Levels of NAD

The maximum contribution of mitochondrial NADH to the fluorescence elicited by 365 mμ light varies among the three preparations studied. Tables I and II summarize this fraction of the signal as the difference between state 5 and state 2. In the case of isolated kidney mitochondria about 40% of the total fluorescence in the anoxic state is derived from respiratory NADH. For the toad muscle mitochondria it amounts to approximately 30%. Too much significance should not be attached to these numbers since changes in excitation and detection filter bandwidth severely affect the relative contributions of NADH and background fluorescence. The differences in fluorescence intensity in the various steady states have, therefore, been expressed as percentages of the state 5-state 2 signal. From Tables I and II it is clear that these levels are closely comparable to the ones defined spectrophotometrically by Chance and Williams (1955 *b*). Thus it is especially significant to note that the state 3 level of NAD reduction determined spectrophotometrically as 50% is in very close agreement with the 42% found fluorometrically for isolated mitochondria and the 42-47% for those within the intact muscle. This agreement facilitates interpretations by extrapolation from the in vitro to the in vivo situation.

It is of some interest that for all practical purposes iodoacetate gives rise to a functionally complete blockage of substrate provision to the mitochondria. Sources other than glycolysis appear to be insignificant in their ability to underwrite the substrate demands for recovery metabolism. In the resting state, however, nonglycolytic pathways are capable of supplying the necessary substrate levels when the temperature is relatively low (11-13°C).

The behavior of the fluorescence trace during changes from one state to another is with two exceptions in full accord with expectations from spectrophotometric measurements on isolated mitochondria and intact muscles. The state 5 level of fluorescence was found to be significantly higher than the state 4 signal, whereas spectrophotometrically no differences in NAD reduction were noted (Chance and Williams, 1955 *b*). However, this disagreement is probably not serious since it could easily arise either from tissue differences or from differences in the substrate supplied. A more important difference exists between the present fluorometric results on recovery metabolism and those from spectrophotometric measurements of NADH in intact muscle (Jöbsis, 1963 *b*). In the spectrophotometric experiments the cycles were absent or small with a rapid return to the base line. The difference clearly arises from the enhancement of mitochondrial NADH fluorescence; for all practical purposes, glycolytic NADH does not contribute to the signal. Under conditions in which gly-

colysis was heavily curtailed, such as in the aged toad sartorii, similar cycles for NADH could be observed spectrophotometrically (Jöbsis, 1963 *b*). Thus it is clear that the loss of spectrophotometric signal stems from equal and opposite changes in mitochondrial and glycolytic NAD fractions, a situation that is avoided in fluorometry.

In the experiments described in this paper the influence of glycolysis was noticed only as a curtailment of the extent of the fluorescence cycle (following paragraphs) and as oscillations of the respiratory chain NADH. Oscillations in glycolytic chain NADH were observed in the very first fluorometric observations on the reduction level of intracellular NAD by Duysens and Amesz (1957). In one of their figures and in their discussion they noted that upon the occurrence of anoxia the NADH fluorescence from yeast cells increases and goes through a damped oscillation before settling down to a new steady level. This observation was extensively verified and expanded by Ghosh and Chance (1964) and Hommes and Schuurmans Stekhoven (1964). Examination of glycolytic intermediate and nucleotide levels showed fluctuations in these components which were either lagging or leading the NADH changes. Earlier suggestions that ADP might play a key role in the production and/or maintenance of the oscillations have been substantiated by the extensive studies of Betz and Chance (1965 *a*, *b*). From the fluorometric measurements presented in this study (see Fig. 11) it is clear that the sudden production of high levels of ADP or P_i resulting from contractile activity can also initiate oscillations. Since oscillations in glycolytic NADH cannot account for the oscillations in fluorescence observed in toad muscle, it is concluded that accompanying changes in ADP must be responsible for the oscillations observed in the respiratory NADH. The possibility of a direct reduction of $NAD_{(2)}$ via α-glycerophosphate arising after glycolytic activity, was rejected because of quantitative considerations (cf. p. 1027).

Studies on the maximum degree of oxidation of mitochondrial NADH produced by contractile activity showed that the effects of the number of twitches on the fluorescence intensity followed a typical saturation curve. The maximum degree of saturation, designated the P_{max}, was shown to be dependent on the twitch rate. At rates slower than one every 3 sec a lowering of the ADP level takes place between twitches as indicated by the lower P_{max}. Conversely it was shown that at rates of one per sec the activity produced a maximal degree of steady-state level change of the mitochondrial NADH. In this way the steady-state level for the recovery metabolism (state 3) was found (see Table II). However, this also emphasizes that the P_{max} is not the parameter of choice to indicate the total amount of recovery unless great care is taken in the construction of a "standard" curve for each muscle of the P_{max} as a function of the number of twitches (Jöbsis, 1964 *b*).

From the qualitative and quantitative conformity of the labile fraction of

the whole muscle fluorescence to the steady-state oxidation-reduction levels of mitochondrial NADH, it is concluded that this fraction of the signal derives from the mitochondrial NADH.

The Fluorescence Cycle As a Quantitative Index to Recovery Metabolism

At this point a note of comparison between these recovery cycles and those of earlier investigators is of interest. The shape of the simple monotonic fluorometric cycles conforms quite satisfactorily to other measurements of oxidative recovery metabolism. Hartree and Hill noted in 1922 that in frog sartorii at 0°C the maximal rate of recovery heat production was reached about 3 min after the completion of a tetanus and that the length of the tetanus was immaterial. At 20°C, however, the peak was found to be dependent on the length of the tetanus; 0.06, 0.12, and 0.50 sec of stimulation provided maximal rates of recovery heat production at approximately 50, 40, and 20 sec, respectively. In addition, Fischer (1930) showed curves with much the same time relations for oxidative recovery in both frog and mouse muscles.

In 1928 Fenn measured the O_2 uptake in recovery metabolism with sufficient accuracy to estimate its time course. Inspection of his published records shows that the rate is not immediately maximal. The peak is delayed by perhaps a minute or two. The records of D. K. Hill (1940) show the same features: the maximal rate of O_2 uptake by a frog sartorius at 0°C occurs some 2 or 3 min after a 12 sec tetanus.

In the present study the peak of the fluorometric response is reached approximately 30 sec after twitch activity at 12°C. Little, if any, shift towards an earlier occurrence is evident at higher temperatures. In the response to large numbers of twitches or tetanic stimulation of varying durations (unpublished experiments) a tendency is noted towards an earlier occurrence of the peak. This shift agrees with the heat data at 20°C and is caused mainly by the onset of the peak-and-plateau type of response rather than by an increase in the final return to the base line. Thus, in general, the agreement between the present measurements and previous ones on oxidative recovery metabolism appears satisfactory. The slightly different time relations between the data presented here and the findings from respiration and heat measurements are probably related to species and temperature differences.

The time course of the onset of the fluorescence response, i.e. the rate of NADH oxidation, is mainly a function of respiratory chain kinetics. Upon the addition of ADP to toad muscle mitochondria, the oxidation of NADH is relatively slow compared to that of other preparations; e.g., rat kidney mitochondria. The time to P_{max} falls between 10 and 15 sec for the former and is about half that value for the latter type of preparation. A partial permeability barrier could account for these observations, although a difference in reaction rates would appear to be a more straightforward explanation.

The peak-and-plateau cycles are strongly reminiscent of the behavior of cytochrome *b* as measured spectrophotometrically (Jöbsis, 1963 *b*) but the factors underlying this type of response are not entirely clear. It was never encountered in the mitochondrial preparations. From a standpoint of ~P bond metabolism the shape signifies a momentarily high level of ADP followed by a longer lasting lower level. A tentative explanation can be presented as follows. Immediately after the contraction the ADP level is high and the mitochondrial NADH responds with a rapid rate of oxidation. This process becomes limited by the concomitant ADP-diminishing action of the transphosphorylating systems. The peak at 20-30 sec signifies the temporary balance of the two effects on NADH. Subsequently, however, the ~P buffering systems continue to decrease the ADP level until the final equilibrium concentration has been achieved. This lower ADP concentration is signified by the diminished excursion of the trace; i.e., the plateau level. The above scheme appears to be at variance with other limits set on the speed of the creatine kinase and myokinase reactions (Carlson and Siger, 1960; Cain and Davies, 1962). However, it should be kept in mind, that even the peak indicates only a few per cent of the ADP level that would occur in the absence of the transphosphorylating systems (Jöbsis, 1963 *b*, and following paragraphs). Thus the last stages of this buffering function are still highly significant. At present, the direct analytical methods are unable to distinguish such small differences in concentration.

Both the peak and the area of the fluorescence cycles provide a basis for the quantitative comparison of different types and amounts of activity. In addition the height of the peak can be used as a means to calculate the amount of ATP hydrolyzed in one twitch. Comparison of the apparent K_M for ADP of the mitochondria in state 4 (Fig. 2) with the number of twitches required to produce a half-maximal state 4-state 3 transition in the intact muscle (Fig. 13) provides an estimate of the rise of intracellular ADP per twitch. In experiments between 10 and 12°C the toad muscle mitochondria showed a K_M for ADP of 14-16μM. At this temperature the half-maximal fluorometric effect was produced by approximately two twitches. Calculated in this way the average value varied between 6 and 9 μmoles/kg per twitch. Less extensive experiments at 24°C suggested a slightly smaller amount (5-7 μmoles/kg). However, the number of experiments at the higher temperature was too small to ascertain the significance of this difference. Similar calculations from spectrophotometric measurements gave a value of 5 μmoles/kg (Jöbsis, 1963 *b*).

These numbers should be compared with observations and calculations based on direct biochemical analysis. Such experiments have shown a hydrolysis of ATP of 0.3-0.4 μmoles/g per twitch (Carlson and Siger, 1960; Infante and Davies, 1962). Taking the phosphocreatine stores into account, a breakdown of this amount of ~P would be buffered to such an extent that the

intracellular ADP level would increase by only 2-3 μmoles/kg per twitch (Jöbsis, 1963 *b*). The general agreement between this calculation and the estimates from spectrophotometric and fluorometric observations is satisfactory.

From Fig. 3 it is clear that the area under the fluorescence cycle, the $\int\Delta Fl$, is linear with the amount of ADP added. This finding which appears intuitively reasonable has been substantiated by more formal considerations. These will be published elsewhere in some detail. A summary must suffice for the present argument.

The oxygen utilization reaction involves 11 components of the respiratory chain proper and is obligatorily coupled to the reactions of oxidative phosphorylation which comprise at least three more enzymatic steps at each of three sites. The reactants for this function are substrates, oxygen, ADP, and P_i. Since many, and perhaps all, the above reactions are reversible under some conditions, the complete reaction scheme of the system is a thing of great complexity. The addition of ADP to mitochondria in state 4 intiates an oxidation cycle of NADH. It seems almost impossible to present the underlying kinetics in such a form as to allow the formal computation of the integral of the reactions underlying this cycle and so this has not been attempted.

Considerable simplification of the reaction scheme allows us to consider that the redox reaction between NADH and oxidized flavoprotein is blocked in state 4 due to the combination of NADH with an unknown inhibitor to form a compound, NADH~I, which cannot react with the flavoprotein subsequent to it in the chain. Upon the addition of ADP, phosphorylation reactions result in the turnover of an equimolar amount of NADH~I to NADH which reduces the flavoprotein and becomes oxidized itself until subsequently it is returned to NADH~I with a concomitant utilization of oxidizable substrate. Since it has been shown that the rate-limiting step is the transfer of the reducing equivalents rather than the oxidative phosphorylation reaction (Chance and Williams, 1956), and since under these circumstances the flow of reducing equivalents is irreversible, the scheme can be greatly simplified. Several simplified schemes have been tested. In these the NAD^+ formed and reduced again should be considered as the "intermediate", the concentration of which is indicated by the amount of fluorescence lost.

A limited number of formal mathematical as well as analog computer studies have been performed and further work is still being carried on. From the preliminary results it appears that in unbranched, consecutive reaction systems the time integral of the concentration of an intermediate is directly related to the amount of primary reactant added. This was found to hold true regardless of the order of the reaction and the reversibility or irreversibility of the steps. A full account of these and similar studies will be published separately upon their completion.

Although still incomplete, these efforts lend some additional confidence to the experimentally determined relation between the amount of added ADP and the $\int\Delta Fl$. Furthermore, it is of great practical value that the same relation holds for different base levels of the initial steady state as was shown in the experiments with the Mg^{++}-activated ATPase.

It has been shown repeatedly that the $\int\Delta Fl$ of intact muscles is linear with the contractile effort over a three- to fivefold range without benefit of glycolytic inhibitors (see for instance Figs. 8 and 14). Beyond this number of individual twitches glycolytic conversion of ADP to ATP begins to play an increasing role in the recovery metabolism at 10-12°C. At higher temperatures the linear range appears to be shorter. Figs. 8 and 14 are but two examples of a large number of similar experiments from which it became evident that the $\int\Delta Fl$ of intact muscles increases linearly with the contractile activity, at least for series with limited numbers of twitches. With greater numbers of twitches deviations from linearity occur again even in the presence of IAA. This is ascribed to the catabolic removal of AMP as was shown earlier by Cain and Davies (1962) by direct, biochemical analysis.

Oxidative Phosphorylation and Glycolysis in Recovery Metabolism

From experiments with and without IAA it was concluded that glycolysis is activated to a significant degree at about five twitches at a rate equal to or faster than two twitches per minute. At very rapid rates (1-0.3 cps) recovery via oxidative phosphorylation appears to be only part of the total recovery metabolism.

Since a series of four twitches at 1.0 cps is the smallest one to show a deviation from the linearity between $\int\Delta Fl$ and number of twitches, a threshold level of ADP or P_i can be calculated for the activation of glycolysis. From the P_{max} saturation curves it was concluded above that at 10-12°C one twitch increased the intracellular ADP concentration by 6-9 μmoles/kg. Thus a level of 24-36 μmoles is just sufficient to activate the glycolytic chain. Indications are that at higher temperatures this threshold level would be substantially lower: at 24°C perhaps one-half to one-quarter of the level at 10-12°C.

These results are in good agreement with the spectrophotometric measurements on acidification due to glycolytic activity (Jöbsis, 1963 *b*). From the color changes of an intercellular pH indicator (bromcresol purple) it was found that lactic acid production was initiated by four and probably by even two twitches. These results were obtained on frog sartorii at 18°C. Apparently the glycolytic system of the toad sartorius at 10-12°C is only slightly less easily activated. All these experiments were performed with adequate oxygen supply. Anoxia can definitely be ruled out under these conditions as proven by spectrophotometric observations on cytochromes *a* and a_3 (Jöbsis, 1963 *b*).

Comparable analytical data on lactate formation have not come to the

attention of the authors. The production of lactic acid under anoxic conditions has been studied extensively over the last 60 yr. Apparently, however, no studies are available for very small numbers of contractions. For anoxic conditions Karpatkin et al. (1964) showed that after 5 min of activity at a rate of 48 twitches per minute intracellular lactate was increased in the frog sartorius at 20°C. However, this earliest point yielded a proportionately lower lactate concentration than the analyses after longer periods of stimulation; i.e., a definite lag period is present. Danforth and Helmreich (1964) found somewhat similar relations for the conversion of phosphorylase *b* to *a* in response to anoxic twitch activity in frog sartorii. At 20°C a lag period of some 25 sec was present when stimulation occurred at 2 cps. However, the lactate level may have been elevated very slightly after about 16 contractions, the first point of their analyses. At a more rapid rate (6 cps) the lag period is curtailed, the curve rises more steeply, and after some 3-4 sec there is some increase in the percentage of phosphorylase *a*. Thus two aspects of the anoxic glycolytic system (lactate formation and phosphorylase *b* kinase activity) have been found to be stimulated by relatively intense amounts and rates of contractile activity. The present study shows that under norm-oxic conditions glycolysis is stimulated after some four to five twitches. This observation extends the range of successful inquiry to the lowest activity possible.

The concept of a lag period (Karpatkin et al., 1964) still appears valid, although it might be preferable to take the point of view that a threshold quantity of some by-product or end product of contraction is required to initiate glycolysis.

From the fact that the phosphorylase *b* to *a* transition is not affected by the work performance, but only by the rate and number of twitches, Danforth and Helmreich (1964) concluded that energy turnover does not seem to be the controlling factor. If so, this would tend to imply some constant factor released by each action potential, such as Ca^{++}. From the work of Rothschld (1930) it is known, however, that energy turnover rather than a mere number of contractions controls lactate formation. Therefore, these two aspects of the glycolytic system are apparently controlled by different activity parameters. The results presented in this study do not provide conclusive evidence concerning which of these controls dominates in the very early activation of glycolysis. Preliminary evidence favors an ADP dependence, in which case a direct activation of the glycolytic enzymes would be implicated.

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