

# Fluorometric Studies of Oxidative Metabolism in Isolated Papillary Muscle of the Rabbit

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**ABSTRACT** The fluorometric technique for measuring the levels of reduced pyridine nucleotides was used to study oxidative metabolism in isolated rabbit papillary muscle at 23°C. The 100% standard level of tissue fluorescence was defined as that measured for muscles resting in oxygenated 10 mM pyruvate solution. This level increased 15% with anoxia and decreased 45% with stimulation in substrate-free solution. Thus, about one-half of the standard tissue fluorescence was metabolically labile and this labile fraction is suggested to be mitochondrial in origin. Decreased tissue fluorescence following mechanical activity was identified with increased oxidation of mitochondrial reduced nicotinamide adenine dinucleotide (NADH) owing to stimulation by adenosine diphosphate (ADP), released during activity, of mitochondrial respiration. The kinetics of the fluorescence transients were slowed fourfold by removal of pyruvate. This effect was not significantly reversed by addition of 10 mM glucose. The time integrals of the fluorescence transients were linearly related to the amounts of mechanical activity in the presence, but not in the absence, of pyruvate. A positive correlation was observed between the steady-state peak tension at constant stimulus rate and the resting level of reduction of pyridine nucleotides in various media. The fluorometric results are interpreted to be indicative of the steady and transient states established by the substrate dehydrogenases and the respiratory chain during oxidative phosphorylation in mitochondria.

## INTRODUCTION

The fluorometric technique for measuring the reduction level of nicotinamide adenine dinucleotide (NAD) in intact tissue (Chance and Jöbsis, 1959; Chance et al., 1962) has been established as a tool for quantitative studies of energy metabolism in amphibian skeletal muscle (Jöbsis and Duffield, 1967 *a*, *b*). Although the technique has also been used as a descriptive adjunct to biochemical studies of the entire heart (e.g., Williamson, 1965; Williamson

and Jamieson, 1966), it has not hitherto been studied as a possible quantitative index of mechanical activity associated with discrete twitches of cardiac muscle. With regard to the latter function, the fluorometric technique possesses certain advantages over existing myothermic and biochemical techniques for studying energy metabolism: it possesses superior sensitivity and time resolution relative to oxygen consumption measurements; because it is a nondestructive method it has greater reproducibility than techniques for measuring high energy phosphate utilization; in relation to the myothermic technique it has the added feature of yielding information about the intracellular metabolism and its control.

The present investigation has three main aims: (*a*) to measure, and account for, the total metabolically labile fraction of tissue fluorescence in isolated papillary muscle; (*b*) to describe the nature of the fluorometric transients following small numbers of twitches; (*c*) to establish conditions under which the fluorometric transients display a quantitative relationship with the amount of contractile activity. Part of this work was included in a recent communication (Chapman and Jöbsis, 1971).

#### METHODS

Beating hearts were isolated from stunned rabbits and perfused with warm physiological saline solution to remove all of the hemoglobin. Papillary muscles from the right ventricles were tied with Measuroil 4-0 silk thread (American Cyanamid Co., Davis & Peck Div., Danbury, Conn.) at each end and mounted vertically in an organ bath. The ventricular tie was clamped below while the tendinous tie was attached to a light chain connected to an isotonic lever. The muscle resting length under a force of 0.5 g wt ranged from 3.6 to 6.6 mm, with a mean of 5.1 mm. The muscle mass, measured after blotting the muscle, ranged from 1.7 to 4.4 mg, with a mean of 2.9 mg. The muscle diameter ranged from 0.59 to 1.15 mm with a mean of 0.86 mm (estimated from the ratio of mass to length).

The organ bath had a capacity of 25 ml and was fitted with a quartz window for efficient illumination and observation of tissue fluorescence. The physiological saline solution contained (in millimoles per liter): NaCl, 130; KCl, 5.0; NaHCO<sub>3</sub>, 25; CaCl<sub>2</sub>, 2.0, and was bubbled vigorously with 95% O<sub>2</sub>:5% CO<sub>2</sub>. Various substrates were added as sodium salts at pH 7.4 in 10 mM concentration. The osmotic differences between substrate-free, 10 mM sodium-substrate, and 10 mM glucose solutions were ignored. A recirculating heating and cooling system maintained the organ bath at temperatures between 22° and 23°C. Whenever the substrate was changed the muscle was stimulated at a rate of 0.25/sec for at least 30 min before further measurements were made. When the muscle was deprived of added substrate no measurements were made until both the fluorescence and tension levels had reached new steady states and had maintained them for at least 10 min. This necessitated waiting for periods of from 30 min to as much as 2 hr before records were obtained in substrate-free solution.

The isotonic lever was fitted with strain gauges for recording tension, which was amplified and displayed oscillographically in the conventional manner. The present study was restricted to isometric contractions.

The microfluorometer was basically similar to that described by Jöbsis and Stainsby (1968). Light from a high pressure, water-cooled, 1000 w mercury arc lamp was filtered with a heat filter and a 366 m $\mu$  filter (30 m $\mu$  bandwidth) (Netherer and Hinz, Hamburg, Germany) and was focussed onto the papillary muscle by means of the epi-illumination attachment of a microscope (Leitz, Wetzlar, Germany). Light from a field of 2 mm diameter was collected by a  $\times$  6.5 objective lens and was split just after the ocular lens system with a 95%-5% beam splitter. The 95% fraction was passed through a  $465 \pm 40$  m $\mu$  widepass interference filter to select the fluorescent light which has a maximum in the 465 m $\mu$  region. The 5% fraction was passed through a 366 m $\mu$  filter to select the reflected excitation light. The fluorescence and reflectance were each detected with an end window photomultiplier tube. The ac output of these tubes was demodulated and the resulting dc voltages were amplified and displayed on an oscillograph. The difference, fluorescence minus reflectance, was also displayed oscillographically and recorded on magnetic tape together with the isometric tension. Feedback regulation of the high voltage supplying the photomultiplier tubes (Jöbsis and Stainsby, 1968) was not used in this work. When necessary, the average of several responses was obtained using a Nuclear-Chicago Model 7100 Data Retrieval Computer (Nuclear-Chicago, Des Plaines, Ill.) coupled to a Hewlett-Packard 7004A X-Y Recorder (Hewlett-Packard Co., Pasadena, Calif.).

The muscles were stimulated electrically via silver electrodes placed on either side near the muscle (but not in the field of observation) using pulses of 10 v with a duration of 10 msec.

## RESULTS

### I. *Steady-State Conditions*

**FLUOROMETRIC STANDARDIZATION** Because the muscles occupied a variable portion of the field of observation that depended upon the size of the individual muscles, an absolute comparison of the fluorescence of different muscles was not attempted. Instead, the 100% level of fluorescence for each muscle was defined as that obtaining for a muscle resting in oxygenated solution in the presence of 10 mm pyruvate ("pyruvate solution"). Under these conditions the background fluorescence from the muscle chamber contributed less than 4%. This standardization was carried out after an equilibration period of at least 1 hr after the dissection, during which the muscle was stimulated continuously at a rate of 0.25/sec. Hence, for metabolic considerations, the preparations can be regarded as being "insulin free." During the standardization, the fluorescence and reflectance signals were adjusted to be equal so that the difference record was relatively free of artifacts arising from noise inherent in the recording system or from instability in the arc lamp. The quality of this type of compensation, relative to the feedback method (Jöbsis

and Stainsby, 1968), becomes less adequate as the tissue fluorescence deviates from the 100% value.

In most muscles under constant 366 m $\mu$  illumination there was a gradual tendency for the standard level of fluorescence to decrease. This rate of decrease was usually less than 10%/hr and, when necessary, was virtually eliminated by obstructing the incident light beam except when measurements were being made.

As the mean muscle length was 5 mm, the fluorescence could be sampled from only 40% of the muscle length on the average. The choice of the recording locus affected only the absolute value of the tissue fluorescence owing to slight variations of diameter along the muscle. However, all the results to be described in this paper were independent of the recording locus. All subsequent steady-state or transient alterations of tissue fluorescence are expressed as a percentage change of the resting oxygenated pyruvate level, regardless of the medium in which they were measured.

**LABILITY OF TISSUE FLUORESCENCE** Because the tissue fluorescence at 465 m $\mu$  is not specific for reduced NAD (NADH), it is important to estimate what fraction of tissue fluorescence is metabolically labile. To this end various metabolic manipulations were performed to favor maximal reduction of NAD, indicated by an increased fluorescence, or maximal oxidation of NADH, signalled by a decreased fluorescence. The results of these experiments are summarized in Table I where the steady-state levels of fluorescence under various conditions are expressed as percentages of the resting level in oxygenated pyruvate solution. The maximal increase in fluorescence was obtained by switching from 95% O<sub>2</sub>:5% CO<sub>2</sub> to 95% N<sub>2</sub>:5% CO<sub>2</sub>

TABLE I  
STEADY-STATE LEVELS OF FLUORESCENCE AND  
TENSION AMPLITUDE IN VARIOUS MEDIA

Fluorescence levels were obtained in resting state ("Rest"), while beating at 0.25/sec ("Stim"), or during contracture ("Cont"). Tension amplitude was obtained while beating at 0.25/sec. Means and standard errors are expressed as percentage of value in oxygenated pyruvate solution. Number of experiments is shown in parentheses.

Medium	Fluorescence level	Tension amplitude
	%	%
N <sub>2</sub> , 10 mM pyruvate	Rest, 114.5±1.8 SE (7)	—
O <sub>2</sub> , 10 mM pyruvate	Rest, 100.0±0.0	100.0±0.0
O <sub>2</sub> , 10 mM lactate	Rest, 89.8±3.0 (4)	94.5±2.7 (5)
O <sub>2</sub> , 10 mM glucose	Rest, 74.5±2.8 (10)	78.8±3.4 (8)
O <sub>2</sub> , no substrate	Rest, 64.0±2.8 (9)	74.4±2.5 (9)
O <sub>2</sub> , no substrate	Stim, 55.6±2.6 (8)	—
O <sub>2</sub> , no substrate, 0.5 mM IAA	Cont, 60.0±4.2 (4)	—

and amounted to an increase of 14.5%. About one-half of the total tissue fluorescence was metabolically labile as indicated by the lowest value of 55.6% obtained for muscles contracting isometrically at a rate of 0.25/sec in the absence of added substrate. Note that the resting level in 10 mM lactate solution indicates a relatively more oxidized state than in pyruvate solution, and that the amount of oxidation indicated with 10 mM glucose is more comparable with the level in the absence of added substrate than with the levels found in lactate or pyruvate. In muscles stimulated to contracture in the presence of 0.5 mM iodoacetic acid (IAA) and no added substrate, the final fluorescence level was between the resting and stimulated steady-state levels in the absence of substrate without IAA. The effects of substrates on tissue fluorescence were independent of the order in which the substrates were applied, except that IAA poisoning was always irreversible.

**SUBSTRATE EFFECTS ON MECHANICAL ACTIVITY** Table I also shows the steady-state amplitude of isometric tension for muscles contracting at 0.25 beats/sec in the presence of various substrates. The values are given as percentages of the amplitude found in pyruvate solution. A correlation between the steady-state tension amplitude and the resting fluorescence level is apparent. There were no significant differences in resting tension among the various media.

## II. *Fluorometric Transients Associated with Mechanical Activity*

**NATURE OF THE RESPONSE** Fig. 1 shows original records obtained for a single twitch (*a*) and for six twitches at 0.25/sec (*b*) in 10 mM pyruvate solution. The records show, from top to bottom, fluorescence, reflectance, difference (fluorescence minus reflectance), and isometric force. The difference trace was filtered with a time constant of 100 msec; the remaining three traces were unfiltered (time constant = 30 msec). The optical traces contain a large movement artifact associated with each mechanical event. No attempt was made to eliminate or minimize this artifact. In each case the reflectance trace returned to its control value during the diastolic interval, whereas the fluorescence decreased (downward deflection) and either returned exponentially to the baseline or decreased further following a subsequent beat. For the purpose of analyzing the fluorometric waveforms the movement artifacts were ignored by linear interpolation between the diastolic intervals. It was assumed that the errors in performing this interpolation were negligible. Close inspection of the traces in Fig. 1 reveals that the subtraction method removed from the difference trace most of the "spontaneous" small transients and drift evident in the fluorescence and reflectance traces.

For the particular traces illustrated in Fig. 1 the movement artifacts in the fluorescence and reflectance traces differ both in magnitude and direction.

This behavior was extremely variable among different muscles: for example, some muscles would show large reflectance artifacts and almost no fluorescence artifacts. Such variability is to be expected because the reflectance artifact depends mainly on changes in the geometry of the reflecting surface, whereas the fluorescence artifact depends mainly on changes in the light-scattering properties of the tissue and somewhat on the mass in the optical field. However, the diastolic optical changes were totally reproducible between different muscles in that stimulation of a resting muscle always produced a *decrease* in fluorescence, whereas the reflectance remained unchanged.<sup>1</sup>

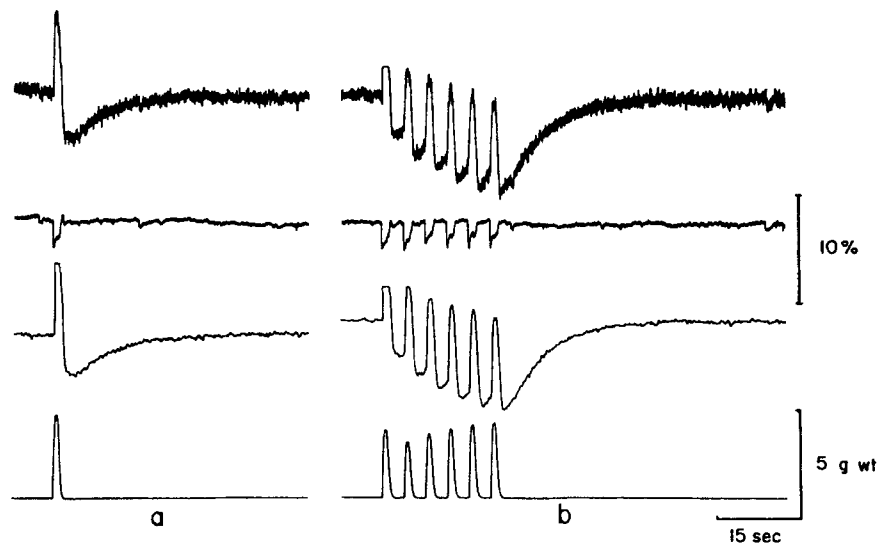


FIGURE 1. Original records of (top to bottom) fluorescence, reflectance, difference, and isometric force for a single twitch (a) and for six twitches at 0.25/sec (b). 10 mM pyruvate solution.

Although only the difference and tension signals were recorded on magnetic tape for averaging purposes, the four traces were always monitored continuously on the oscillograph. Hereafter the difference trace will be referred to simply as fluorescence.

#### KINETICS OF THE RESPONSE

(a) *10 mM Pyruvate Solution* It is clear that the "off kinetics" of the fluorometric transients are amenable to detailed analysis. As noted for Fig. 1, the off kinetics were always clearly exponential in 10 mM pyruvate solution. The

<sup>1</sup>Some muscles were encountered that showed little or no decrease in fluorescence even following many beats. These muscles always showed a distinct yellowish color under 366 m $\mu$  irradiation in contrast to the bright blue fluorescence characteristic of muscles yielding successful experiments. When such muscles were encountered the experiment was abandoned without further investigation.

time constant of decay was independent of the number of beats and of the rate of stimulation for rates of 0.3/sec or less as illustrated by Fig. 2, which shows semilogarithmic plots of the exponential return to baseline of averaged fluorescence traces following various numbers of twitches at different rates. The lines were fitted by eye to the first 8 sec of the graph; thereafter the fit deteriorated as baseline noise became a significant factor. The variation of the time con-

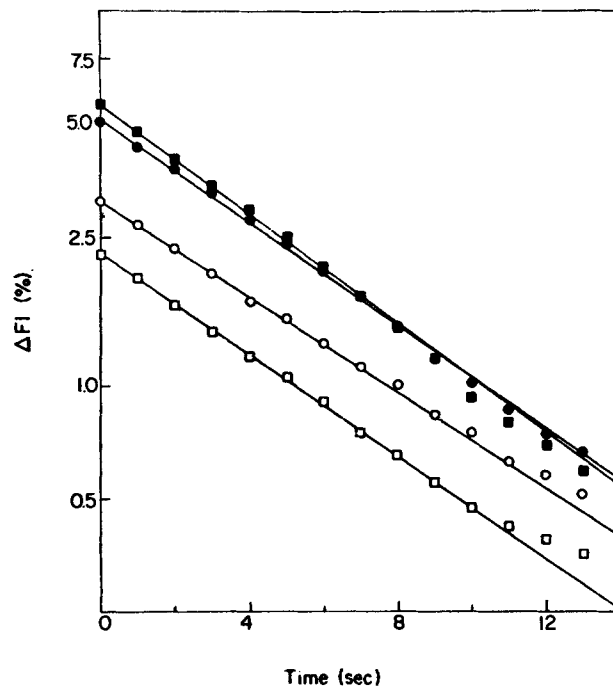


FIGURE 2. Off kinetics of fluorescence transients, averaged over five repetitive runs, obtained in 10 mM pyruvate following a single twitch (open squares), 10 twitches at 0.15/sec (open circles), 12 twitches at 0.25/sec (filled circles), and 16 twitches at 0.30/sec (filled squares).

stant among these four lines was slight, and probably random, compared with the effect of altering the substrate.

(b) *Effect of Substrate Depletion and Glucose* Fig. 3 shows original records of fluorescence and isometric force for a muscle stimulated 20 times at 0.25/sec in 10 mM pyruvate solution (a) and substrate-free solution (b). The oscillations in the fluorescence records were due to movement artifacts. Careful inspection of all three original optical traces (as in Fig. 1) revealed that the lower extremity of each oscillation corresponded to the true diastolic fluorescence signal. As already indicated in Table I, the isometric force was weaker in substrate-free solution than in pyruvate solution and this was correlated with a smaller

amplitude of fluorescence decrease in substrate-free solution. Another consistent feature of the mechanical activity in the presence of pyruvate was the relatively small staircase effect. However, the most striking difference among

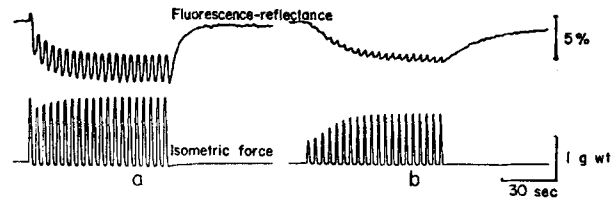


FIGURE 3. Original records of fluorescence and isometric force for 20 twitches at 0.25/sec in 10 mM pyruvate (*a*) and after 110 min in substrate-free solution (*b*).

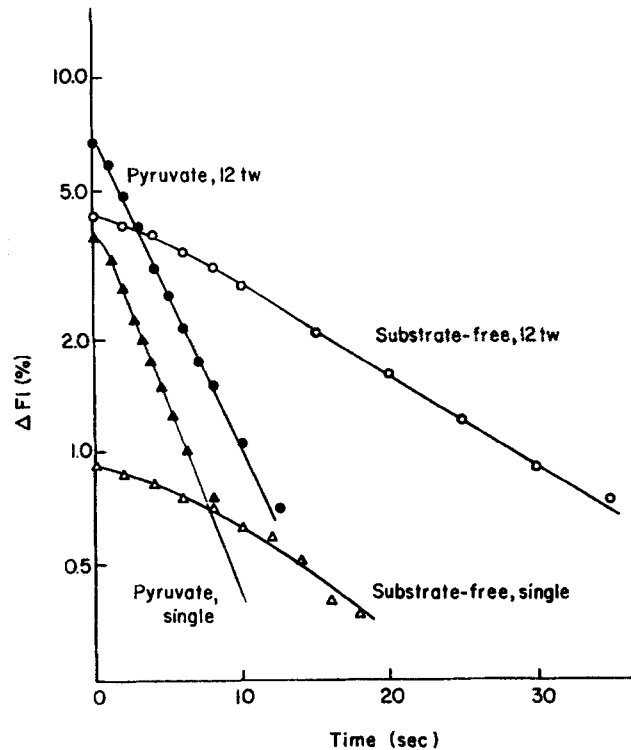


FIGURE 4. Off kinetics for 10 averaged fluorescence transients obtained in 10 mM pyruvate and substrate-free solutions following single twitches and 12 twitches at 0.25/sec.

the fluorometric responses of Fig. 3 was the relative prolongation of the recovery kinetics in the absence of pyruvate. This is illustrated by semilogarithmic plots in Fig. 4 of averaged off kinetics of responses to single twitches and 12 twitches at 0.25/sec for a muscle in pyruvate and substrate-free solutions. The fit of the substrate-depleted off kinetics to an exponential decay was rela-



tively poor, but even the variability between the substrate-depleted curves was slight compared with the over-all effect of removing pyruvate.

The behavior in 10 mM glucose solution was essentially similar to that in substrate-free solution. Table II shows time constants and half-decay times for the off kinetics in pyruvate, glucose, and substrate-free solutions. More experiments than are indicated in Table II have produced similar results, but Table II is restricted to experiments from which averaged responses for at least five identical records were available. All experiments to date have shown that the mechanical and fluorometric effects of removal of pyruvate are relieved little, if at all, by replacement with glucose. Preliminary work with lactate, acetate, and  $\beta$ -hydroxybutyrate indicates that these substrates produce effects intermediate between those of pyruvate and glucose.

TABLE II  
OFF KINETICS OF FLUORESCENCE SIGNALS OBTAINED  
FROM AVERAGED RESPONSES OF AT LEAST FIVE RECORDS

Medium	Time constant	Half-decay time	No. of experiments
	<i>sec</i>	<i>sec</i>	
10 mM pyruvate	6.72±0.46 SE	4.86±0.33	11
10 mM glucose	—	19.0 ±3.2	4
No added substrate	—	20.8 ±2.6	4

**FLUOROMETRIC TRANSIENTS WITH COMPLEX KINETICS** Occasionally, the fluorometric transients associated with several twitches showed undershoots and overshoots such as are illustrated in Fig. 5. This behavior was extremely rare in muscles that had been set up initially in pyruvate solution but the following procedures occasionally induced such complex transients: change of substrate (e.g., glucose to pyruvate or vice versa); raising the temperature to 30°C; administration of 10<sup>-3</sup> g/ml theophylline; stimulation at high rates (greater than 0.5/sec at 22°C). Whenever these complexities arose in a preparation the experiment was abandoned without discarding the previously obtained data. Although all four of the procedures listed above can be expected to produce metabolic disturbances in the muscle, only the last three can be expected to induce tissue hypoxia. Furthermore, when anoxia was introduced by substituting nitrogen the muscles were always unable to maintain a steady tension amplitude following the initial staircase, whereas only theophylline and stimulation at high rates resulted in poor maintenance of tension amplitude. Also, there was no correlation between the sensitivity of a muscle to the above procedures and the thickness of the muscle. Therefore these occasional fluorometric complexities are regarded as indications of metabolic disturbances other than hypoxia. As these complexities were encountered less than 25% of the time, their further elucidation, which is

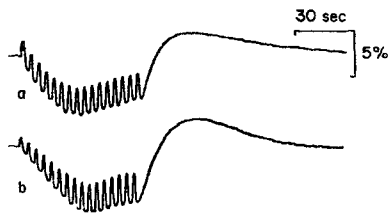


FIGURE 5. Original records of fluorescence transients with complex kinetics for 16 contractions at 0.25/sec in 10 mM pyruvate (a) and 10 mM glucose (b).

peripheral to the main objectives of this paper, is deferred to a later publication.

### III. Quantitative Relation to Contraction

**FLUORESCENCE-TIME INTEGRALS** The fluorescence-time integral ( $\int \Delta F I dt$ ) refers to the area enclosed by the fluorescence transient on a time plot. This quantity has been related linearly to the amount of adenosine diphosphate (ADP) added to isolated mitochondria of skeletal muscle (Jöbsis and Duffield, 1967 *a*) and, for small numbers of twitches, to the amount of mechanical activity of isolated skeletal muscle (Jöbsis and Duffield, 1967 *a, b*). Fluorescence-time integrals were measured by gravimetric analysis, using linear interpolation between the diastolic intervals. Muscles were stimulated for different numbers of twitches from 1 to 20 at a rate of 0.25/sec and the ratio of fluorescence-time integral to the summed tension amplitude ( $\int \Delta F I dt / \Sigma P_o$ ) was found for each number of twitches.

Fig. 6 shows a plot of the ratio  $\int \Delta F I dt / \Sigma P_o$  as a function of the number of twitches for a muscle in pyruvate solution (filled squares) and substrate-free solution (open circles). Each point was obtained from an averaged record of 10 responses. Typically, the ratio was independent of the amount of activity in pyruvate solution, whereas the ratio decreased sharply with increasing amounts of activity in substrate-free solution. The marked dependence of this ratio on the amount of activity in substrate-free solution was also characteristic for 10 mM glucose solution. Hence the fluorescence-time integral can be used as a linear indication of the amount of mechanical activity in 10 mM pyruvate solution at 23°C, for amounts of activity extending up to 20 isometric twitches, without having to use metabolic inhibitors such as iodoacetic acid. This is particularly fortunate as the iodoacetate-poisoned papillary muscle is not a satisfactory preparation for detailed mechanical or energetic studies.

It was always found that the ratio  $\int \Delta F I dt / \Sigma P_o$  was smaller in the presence of pyruvate than without pyruvate, irrespective of the presence of glucose. This was due to the decreased mechanical output and the protracted metabolic recovery kinetics in the absence of pyruvate (see Table I and Fig. 3). The absolute value of the ratio in pyruvate solution varied considerably

among preparations because of the wide variability both in the amplitude of the fluorescence transients (discussed later) and in the tension amplitudes. However, for any particular preparation, the ratio in pyruvate solution was constant within 10% of the mean value for 4–20 twitches. For smaller numbers of twitches (less than four) there was sometimes more than 10% variation. This may have been due to the fact that the staircase phenomenon occurs mainly in the first four twitches in pyruvate solution.

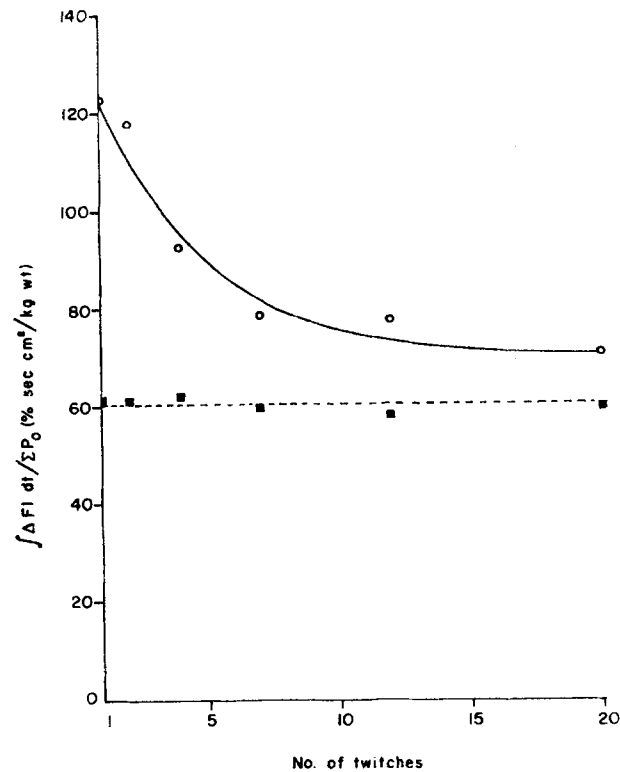


FIGURE 6. Relation of fluorescence-time integral per unit summed tension to number of twitches at 0.25/sec in 10 mM pyruvate (filled squares) and substrate-free solution (open circles). Data were obtained from averages of 10 identical responses.

**AMPLITUDE OF FLUORESCENCE TRANSIENTS** No linear correlation could be found between the amplitudes of the fluorometric transients and the developed tension. The mean developed tension in a block of contractions,  $\bar{P}_o$ , was divided into the maximum fluorescence decrease,  $\Delta F I_{\max}$ , to give the ratio  $\Delta F I_{\max} / \bar{P}_o$ . The mean tension amplitude was obtained by averaging the twitch amplitudes in a block of contractions: this was done to allow for the staircase phenomenon which influenced the amplitude of  $\Delta F I_{\max}$ . The ratio  $\Delta F I_{\max} / \bar{P}_o$  was always markedly dependent on the number of twitches and

this dependence varied according to the presence or absence of pyruvate. Fig. 7 shows typical plots of  $\Delta F_{\text{max}}/\bar{P}_o$  versus the number of twitches for a muscle in 10 mM pyruvate solution (filled squares) and 10 mM glucose solution (open circles). The values for infinite numbers of twitches were calculated from double reciprocal plots of  $\bar{P}_o$  and  $\Delta F_{\text{max}}$  as functions of the number of twitches. It was characteristic for the value of the ratio  $\Delta F_{\text{max}}/\bar{P}_o$  to be lower in glucose solution relative to the value in pyruvate solution for small numbers

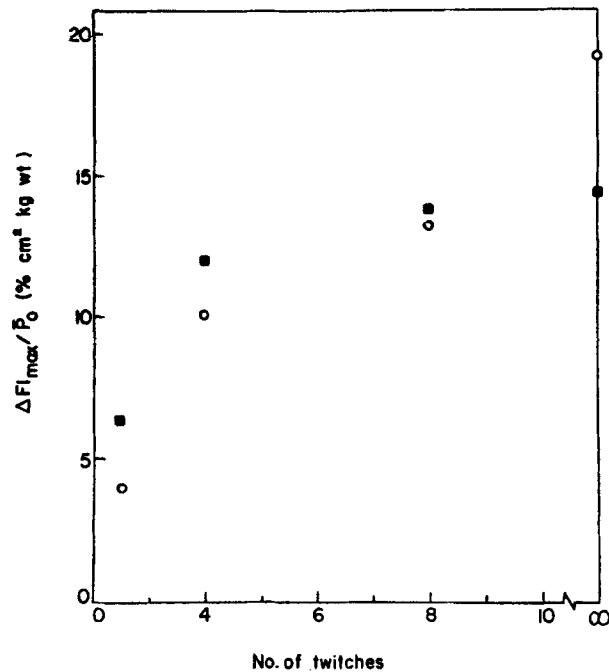


FIGURE 7. Relation of maximum fluorescence decrease per mean twitch amplitude to number of twitches at 0.25/sec in 10 mM pyruvate (filled squares) and 10 mM glucose solution (open circles). Data were obtained from averages of five identical responses.

of twitches. However, this relationship was reversed for large to infinite numbers of twitches. This behavior in glucose was also typical of that in substrate-free solution. One-half of the maximum fluorescence change with pyruvate as substrate was usually achieved after one or two twitches. In the absence of pyruvate it usually required 5–10 twitches to achieve the half-maximum fluorescence changes. The absolute magnitude of the saturation amplitude of  $\Delta F_{\text{max}}$  showed considerable variability among preparations, ranging from about 3% to more than 10% of the standard 100% level in 10 mM pyruvate solution. The saturation amplitude of  $\Delta F_{\text{max}}$  for any given preparation increased with increasing stimulus frequency up to 0.5/sec (unpublished observations, see also Fig. 2)

## DISCUSSION

The first part of this discussion seeks to identify the tissue elements that underly the labile fluorescence of the isolated papillary muscle. In this section it is concluded that mitochondrial NADH is the sole significant origin of labile fluorescence under the conditions used for the present experiments. The second part is devoted to a biochemical interpretation of the different resting fluorescence levels seen in the presence of different substrates. The third part of the discussion concerns the nature and quantitative relations of the fluorometric transients associated with mechanical activity.

*I. Identification of Labile Tissue Fluorescence with Mitochondrial NADH*

Jöbsis and Duffield (1967a) have shown in amphibian skeletal muscle that the tissue fluorescence arising from cytoplasmic NADH is an insignificant fraction of that arising from mitochondrial NADH. One qualitative reason for this is possibly the fact that the cytoplasmic enzyme which most strongly binds NADH, glyceraldehyde phosphate dehydrogenase, is one of the few enzymes which actually diminishes, rather than enhances, the fluorescence of NADH (Velick, 1961). In addition to this qualitative consideration there is good direct biochemical evidence that cytoplasmic NADH is not present in sufficiently large concentrations to contribute appreciably to the labile fluorescence of isolated cardiac muscle. Williamson (1965) reported that isolated rat hearts perfused with 10 mM pyruvate solution contained 583  $m\mu$ mole more NADH per gram dry weight than hearts perfused with glucose solution. By assuming that 75% of the intracellular water is extramitochondrial and that the total cellular NAD is roughly equally divided between mitochondria and cytoplasm, Williamson calculated that the free cytoplasmic NADH concentration was 0.5 and 0.08  $\mu$ M for glucose- and pyruvate-perfused hearts, respectively, in the absence of insulin. Thus he concluded that, under these conditions, "the bulk of the total NADH is located in the mitochondria." It should be noted that this conclusion was made concerning data obtained from hearts being spontaneously at 38°C. These preparations would almost certainly have been metabolizing glucose faster than the resting isolated papillary muscle at 23°C, and so the levels of cytoplasmic NADH resulting from the glyceraldehyde phosphate dehydrogenase reaction could be expected to be even less in the present work.

In the present experiments the lactate minus glucose fluorescence difference was  $\frac{3}{5}$  of the pyruvate minus glucose fluorescence difference (see Table I). This result is also consistent with similar measurements by Williamson and Jamieson (1965, 1966) on the isolated rat heart. By analogy with the biochemical data of Williamson (1965) concerning the pyruvate minus glucose NADH difference, one could expect 10 mM lactate to result in the formation of

an extra 350  $\mu\text{mole}$  of NADH (i.e.,  $\frac{3}{5}$  of 583) per gram dry weight relative to the value for the glucose perfused heart. Now the tissue lactate concentration in glucose-perfused hearts is approximately 1 mM (Fig. 7 D of Williamson, 1966), and so one would expect roughly a 10-fold increase in the tissue lactate/pyruvate ratio on switching to 10 mM lactate solution (neglecting the formation of extra pyruvate via lactic dehydrogenase). Hence, at the most, one would expect a 10-fold increase in the cytoplasmic NADH concentration, from a value slightly less than 1  $\mu\text{mole/g}$  dry wt in the presence of glucose (calculated by Williamson, 1965) to a value somewhat less than 10  $\mu\text{mole/g}$  in the presence of 10 mM lactate. This means that less than  $\frac{1}{35}$  of the lactate minus glucose fluorescence difference could arise from the formation of cytoplasmic NADH. This estimate becomes even smaller if the lower fluorescence efficiency of cytoplasmic NADH (see above), and the formation of excess pyruvate from the added lactate via lactic dehydrogenase, are taken into account.

Thus, while the present measurements of the various steady-state levels of tissue fluorescence are essentially consistent with the results of Chance, Williamson, et al. (1965), and Williamson and Jamieson (1965, 1966), the present interpretation is different in that a significant contribution to the fluorescence from cytoplasmic NADH is excluded.

Within the mitochondria there are three likely sources of pyridine nucleotide fluorescence: (a) reduced nicotinamide adenine dinucleotide phosphate (NADPH), (b) NADH associated with reductive biosynthesis (the NADH<sub>(2)</sub> fraction—see below), and (c) NADH associated with oxidative phosphorylation (the NADH<sub>(1)</sub> fraction—see below).

(a) The contribution of reduced nicotinamide adenine dinucleotide phosphate (NADPH) to tissue fluorescence is a significant factor in cardiac muscle. Although the total NADP + NADPH is less than 10% of the total pyridine nucleotide content (Glock and McLean, 1955; Williamson, 1965), the levels of NADH and NADPH are approximately equal in the glucose-perfused rat heart (Williamson, 1965). The formation of NADPH probably accounts for one-fifth of the glucose minus pyruvate fluorescence difference in the rat heart (Williamson, 1965) and must be assumed to make a similar contribution to the steady-state levels in the present work. However, the kinetics of NADPH changes are probably too slow to form a significant contribution to the kinetics of fluorescence changes associated with small numbers of twitches (Chance, Schoener, et al., 1965; Jöbsis and Duffield, 1967 *a*). Hence, the differences in various steady-state levels of fluorescence shown in Table I are regarded as being due to differences in the level of mitochondrial NADH with a small but significant steady-state contribution from differences in NADPH levels. This latter contribution is unlikely to exceed one-fifth of that from NADH.

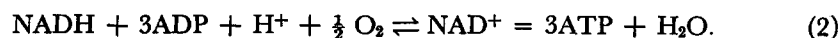
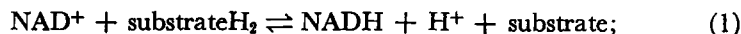
(b) The possible involvement of the mitochondrial NADH<sub>(2)</sub> fraction

(Chance and Hollunger, 1961) in changes in tissue fluorescence has been shown to be insignificant for toad skeletal muscle (Jöbsis and Duffield, 1967 *a*). As the most likely role for the NADH<sub>(2)</sub> fraction is probably in reductive biosynthetic reactions (see Jöbsis, 1964), its involvement in the present results is unlikely to be more significant than in skeletal muscle.

(*c*) Therefore, it must be the NADH<sub>(1)</sub> fraction which underlies most of the labile tissue fluorescence reported in the present work. The NADH<sub>(1)</sub> fraction is the fraction most closely involved in oxidative phosphorylation and thereby in the recovery metabolism of the cell. Thus the monitoring of the redox state of NADH<sub>(1)</sub> by fluorometry provides a measure of energy turnover.

## II. *Steady-State Conditions*

The resting level of mitochondrial NADH depends on the dynamic steady state established between the inflow of reducing equivalents arising from the metabolism of endogenous lipid, carbohydrate, or exogenous substrate (reaction 1), and the flow of electrons along the respiratory chain from NADH to molecular oxygen. The latter reaction is shown greatly simplified as reaction 2:



Reaction 2 is somewhat reversible for certain portions of the respiratory chain. Although a genuine liberation of O<sub>2</sub> at the expense of ATP has not yet been shown, it is known that the mitochondrial level of NADH is also strongly determined by reversed electron transfer, as exemplified by the energy-linked reduction of NAD in the presence of succinate and ATP (Chance and Hollunger, 1961). In the resting muscle, electron flow would be limited by the availability of ADP for oxidative phosphorylation arising from the basal energy utilization (e.g. for active transport processes). Muraoka and Slater (1969) have shown that in resting metabolism the reactions in the respiratory chain approach equilibrium conditions. Thus the net velocities of reactions 1 and 2 would be low in the resting state, and the resulting level of NADH would be determined mainly by the equilibrium constants. Hence, in the present experiments, where succinate was not used, the variation of the resting NADH levels in the presence of different substrates would reflect primarily differences in the equilibrium established with NADH by reaction 1 for the different substrates.

It is of interest that removal of pyruvate produced an effect which was little reversed by substitution of glucose. This finding is consistent with biochemical studies indicating that 75% or more of oxidative metabolism in the isolated rat heart perfused with glucose or no added substrate is underwritten by

endogenous lipid (Fisher and Williamson, 1961; Williamson, 1962; 1965), whereas 10 mM pyruvate, when present, underwrites over 90% of the oxidative metabolism (Williamson, 1961; 1964). (Note that the present experiments and discussion are concerned with insulin-free preparations.) Hence, it may be deduced that 10 mM pyruvate establishes a near-equilibrium with relatively large amounts of NADH, whereas endogenous lipid equilibrates with relatively small amounts of NAD in the reduced form either directly via the acyl dehydrogenases or indirectly via the citric acid cycle. This conclusion is supported by the direct biochemical analysis for tissue NADH in glucose- and pyruvate-perfused rat hearts (Williamson, 1965). The supposition that endogenous lipid equilibrates with low concentrations of NADH is also in accord with the finding of Williamson (1965) that 10 mM acetate produced a barely significant increase in tissue NADH relative to that found in glucose-perfused rat hearts. This finding also suggests that it is the citric acid cycle that dominates the near-equilibrium with mitochondrial NADH in the absence of pyruvate because acetate is utilized directly by the citric acid cycle without the involvement of the acyl dehydrogenases of lipid metabolism. Hence it is suggested that the equilibrium in the presence of pyruvate is dominated primarily by the pyruvate dehydrogenase reaction and not by the subsequent reactions of the citric acid cycle.

10 mM lactate produced resting fluorescence levels intermediate between those produced by glucose and pyruvate. Presumably the lactic dehydrogenase reaction is so far displaced towards lactate (Hohorst et al., 1961) that the pyruvate arising therefrom in 10 mM lactate solution is insufficient to dominate entirely the resting near-equilibrium established with mitochondrial NADH. The greater oxidation of NADH evident in stimulated, substrate-depleted muscles, relative to those stimulated to contracture with 0.5 mM IAA, may not be significant in view of the multiple actions of IAA in this preparation (e.g., endogenous lipid metabolism was probably affected).

It should be noted that the interpretation of the different *resting* levels of NADH is viewed primarily within the framework of the near-equilibria of reactions 1 and 2 pertaining to the resting steady state. For the interpretation of the fluorometric transients associated with activity (see below) it is necessary to consider the influence of the availability of reducing equivalents and of the absolute rates of the reactions concerned.

#### CORRELATION BETWEEN MECHANICAL AND FLUOROMETRIC STEADY STATES

It is well known that calcium ions are transported by mitochondria and that this transport is linked to an oxidation of the respiratory chain components in isolated mitochondria (Chance, 1965; Lehninger et al., 1967). It is also established that mitochondria accumulate  $\text{Ca}^{++}$  in intact skeletal and cardiac muscle and a dynamic role has been postulated for mitochondria in the uptake



and release of calcium ions in vivo (Carafoli et al., 1969; Ueba et al., 1971). Hence, the correlation between the mechanical and the fluorometric data of Table I may be due to a possible influence of the redox state of mitochondria as a modulator of normal excitation-contraction coupling. The cellular level of adenosine triphosphate (ATP) does not seem to be directly affected by the presence or absence of pyruvate as indicated by the tissue analysis of Williamson (1965). Hence, it is unlikely that ATP limitation underlies the correlation of Table I. However, the present finding that mechanical activity was stronger in pyruvate than in glucose solution is in conflict with earlier work on whole hearts and isolated atria at different temperatures (Nakamura et al., 1949; Webb and Hollander, 1956; Gimeno et al., 1966); the reasons for these discrepancies remain obscure.

It should be noted that the release and uptake of  $\text{Ca}^{++}$  by the sarcoplasmic reticulum occur within the time-course of the mechanical event and so the diastolic time-course of the fluorometric transient cannot be significantly affected by the  $\text{Ca}^{++}$  transients (cf. Jöbsis and O'Connor, 1966; Jöbsis and Duffield, 1967 *a*).

### III. *Fluorometric Transients*

With the exception of the occasional responses with complex kinetics (see Fig. 5), the fluorometric transients reported in this paper correspond to the stimulatory effect of extra ADP liberation on mitochondrial respiration, classically described by Chance and Williams (1955, 1956). Thus, the fluorometric transients are indirect records of ADP transients and the speed of the "off" kinetics is a measure of the ability of the respiratory chain to consume the excess ADP by the process of oxidative phosphorylation. The slowed kinetics in the absence of pyruvate may be due partly to the limited availability of endogenous substrate and partly to the slower reactivity of ADP with a more oxidized respiratory chain. Computer simulation of this problem (Chapman, unpublished observations) has indicated that a more oxidized respiratory chain is less sensitive to ADP in that it requires a higher ADP concentration to maintain a given net rate of oxidative phosphorylation. This point is indicated qualitatively by inspection of reaction 2 above in that, for oxidative phosphorylation to occur, ADP must react with reduced components of the respiratory chain. Simulation studies have also indicated the accumulation of larger amounts of creatine phosphate in the resting state in pyruvate relative to that in the absence of pyruvate (Chapman, unpublished observations).

Although it has been stated previously that the oxygen consumption rate of the isolated perfused rat heart is little affected by the nature of the substrate (Fisher and Williamson, 1961; Williamson, 1962), it should be noted that this applies to hearts maintaining a constant level of resting or active metabolism.

In the present situation, where previously resting muscles undergo small numbers of contractions (less than 20) it would appear that the onset of oxidative phosphorylation is slower (see Fig. 7) and that the subsequent recovery ("off kinetics") is more protracted when the respiratory chain is more oxidized, as in the absence of pyruvate.

**QUANTITATIVE RELATION TO CONTRACTION** The finding that the fluorescence-time integral bears a linear relationship to the amount of contractile activity in 10 mM pyruvate solution is consistent with the fact that 10 mM pyruvate, when present, underwrites over 90% of cardiac oxidative metabolism (Williamson, 1961; 1964). Thus, with 10 mM pyruvate the availability of reducing equivalents to the respiratory chain depends on a single metabolic pathway provided with a constant substrate concentration. Under these conditions apparently the rate constants of this pathway are not normally increased by successive stimulation above the resting level. However, when a tissue is dependent on glycolysis a very different situation obtains. An increase of the glycolytic rate occurs gradually over the course of several twitches in the toad's sartorius, giving rise to increasing levels of pyruvate (Jöbsis and Duffield, 1967 *a, b*). This is an important factor underlying the manifestation of fluorometric transients with complex kinetics in the skeletal muscle. A similar effect (see Fig. 5) almost surely has the same general origin in the papillary muscle, but since this tissue does not solely depend on glycolysis the situation may be more complex.

The following factors may be involved in producing the nonlinear correlation between the fluorescence-time integral and the amount of contractile activity in the absence of pyruvate:

- (a) an increase in the availability of reducing equivalents by activation of the enzymes for fatty acid oxidation;
- (b) an increase in the availability of reducing equivalents by activation of glycogenolysis, possibly by  $\text{Ca}^{++}$ - or cyclic AMP-induced stimulation of phosphorylase, or by the effect of adenine nucleotides or inorganic phosphate on phosphofructokinase or glyceraldehyde phosphate dehydrogenase (Wu, 1959; Wu and Racker, 1959; Passonneau and Lowry, 1964; Danforth and Helmreich, 1964; Krebs et al., 1966; Williamson et al., 1967 *a, b*; Scrutton and Utter, 1968);
- (c) attenuation of the ADP levels directly by activation of glycogenolysis activated by the above mechanisms.

The factors listed under (b) are likely to be the most significant causes of the nonlinear correlation in this preparation. The formation of excess cytoplasmic NADH as a result of increased glycogenolysis is not likely to be a significant factor in altering the fluorescence transient (see earlier discussion).

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