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HEAT PRODUCTION AND FLUORESCENCE CHANGES OF TOAD SARTORIUS MUSCLE DURING AEROBIC RECOVERY AFTER A SHORT TETANUS

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SUMMARY

1. The time course of the aerobic recovery following a 0·5 sec tetanus at 20° C of the sartorius muscle of the toad *Bufo bufo*, equilibrated in bicarbonate-CO_2 Ringer solution, has been followed by recording simultaneously the heat production and the fluorescence excited by ultra-violet light at 366 nm.

2. The fluorescence light emitted in these conditions in the region of 450 nm monitors the state of oxidation-reduction of the nicotinamide-adenine dinucleotides (NAD^+-NADH). After a short tetanus, the cycle evoked consists of an initial increase of the fluorescence (reduction of NAD^+) followed by a long lasting phase of decreased light emission. This includes an early period of oxidation of NADH succeeded by a slow reduction of the NAD^+ formed in excess over the resting state. After iodoacetate, the initial reduction is suppressed.

3. The time course of both fluorescence and heat production may be analysed into a rapid and a slow component by a double exponential model.

4. The time courses of the aerobic recovery heat and of the fluorescence changes are similar after five minutes, but differ in their fast components. IAA significantly increases the rate constants of the fast terms of both monitors.

5. The slow component is mainly related to aerobic processes while the fast one is due to both oxidative and glycolytic reactions occurring simultaneously.

INTRODUCTION

The most evident sign of metabolic activity following contraction has for long been the production of heat in excess over the resting rate during the recovery period. The literature on the delayed heat production of striated muscle has been thoroughly reviewed and added to by A. V. Hill

in 1965. It is clear that this recovery heat is for its greatest part related to aerobic phenomena (Hill, 1913; Hartree & Hill, 1922) and that its time course is similar to the kinetics of oxygen consumption (D. K. Hill, 1940*a*). Nevertheless, it has been difficult to interpret myothermic data in terms of precise biochemical reactions, and some other monitor, more specifically related to a chemical reaction is required. The measurement of the surface fluorescence appeared particularly suitable for this purpose. Chance & Williams (1955) showed that ADP stimulates oxidative phosphorylation in isolated mitochondria, causing an oxidation of the dihydro-nicotinamide-adenine dinucleotide (NADH) to nicotinamide-adenine dinucleotide (NAD^+). As ultra-violet light excites the fluorescence of NADH but not that of NAD^+, a record of the fluorescence variations provides an index of the oxidation-reduction state of the NAD^+-NADH couple. This technique was later successfully applied to isolated tissues; in particular, Chance & Jöbsis (1959) showed that twitches of an excited frog sartorius were followed by a cycle of decreased fluorescence, corresponding to an oxidation of the pyridine nucleotides, indicative of some ADP liberation.

Both the myothermic and the fluorometric techniques are especially suitable for kinetic studies on whole muscle. It was thus tempting to investigate by a simultaneous analysis of the time course of each monitor, whether there is some correlation between them. In spite of the fact that most studies on recovery heat have been done on frog muscles, the experiments reported here have been made on sartorius muscles of the toad *B. bufo*, which appear to have a more highly developed oxidative system than frog sartorii; in toad muscle the fluorescence variations are essentially representative of oxidative processes (Jöbsis, 1963). To estimate the possible influence of the cytoplasmic pyridine nucleotides on the fluorescence cycle, the effect of iodoacetate (IAA), an inhibitor of glycolysis, has also been tested.

The present results show that after the first half minute of recovery, in spite of some evident dissimilarity, the aerobic recovery heat and the simultaneous fluorescence changes follow a common pattern, which may be described by a double exponential curve. Iodoacetate modifies some parameters of the kinetics without altering the general time course of the heat production or the change of fluorescence suggesting that glycolysis contributes to the fluorescence changes as well as to the recovery heat.

Some of these results have been presented in preliminary reports (Godfraind-De Becker & Aubert, 1967, 1968; Godfraind-De Becker, 1968).

METHODS

Physiological material and solutions

The experiments were performed on paired sartorii of toads *B. bufo* purchased from local sources. After dissection, the muscles were mounted on both sides of a thermopile and allowed to equilibrate at 20° C in Ringer solution (mM: NaCl 95, KCl 2·5, $NaHCO_3$ 20, mono- and diacid sodium phosphates 1·9, $CaCl_2$ 1, $MgSO_4$ 1) oxygenated with a gas mixture of O_2 and CO_2 (95:5 v/v; pH 7·3).

During the actual experiment, the solution was replaced by the pure gas mixture. Each series of measurements consisted of three to four tetani at 15 min intervals; to minimize the effects of movement artifacts, the results of the first contraction were always ignored and the analysis was performed on the average of the two or three further tetani. For each pair of muscles, two series were performed, separated by a period of rest of about 1 hr in the oxygenated solution.

In some experiments, the muscles were poisoned with sodium iodoacetate 0·4 mM (IAA) during 30 min before a new series of stimulations.

The experiments reported here were performed between September and February and include only those where heat and fluorescence could be analysed simultaneously.

Force measurements

The muscles were stimulated isometrically for 0·5 sec by alternate condenser discharges at a frequency of 100/sec with a just supramaximal voltage. The force developed was measured by means of strain gauges on a Sanborn Twin-Viso Recorder. It was used only as an indication of the condition of the muscle.

Heat measurements

The thermopile was made of two sets of twenty-seven manganin-constantan couples, connected in parallel and giving 1484 μV/° C at 20° C. The total length of the pile was 22·5 mm, including the stimulation electrodes. As the muscle length ranged from 24 to 28 mm, the heat was measured from almost the whole of the muscles. The e.m.f. arising from changes in temperature within the muscles was fed into a recording galvanometer (Kipp BD 2 Micrograph or Graphirac Sefram after amplification with a Sefram Amplispot).

For the analysis of recovery heat, the delay of heat conduction was of no importance as the thermopile was very thin but the results, calculated in blocks of 0·5 min interval, had to be corrected for the heat losses. The thermopile showed an exponential rate of temperature decay, k. For a time interval Δt, this correction was equal to k times the area under the temperature curve during the interval considered. For short time intervals, this area was usually approximated by the trapeze rule and the correction for heat loss was represented by

$$k\Delta t\left(\frac{V_t + V_{t+\Delta t}}{2}\right),$$

where V_t is the reading at time t. But with Δt as long as 0·5 min, the error involved could be large in the first stages of recovery. A better approximation was given by the use of the logarithmic mean; the correction computed as

$$k\Delta t\left(\frac{V_t - V_{t+\Delta t}}{\ln V_t/V_{t+\Delta t}}\right)$$

was strictly correct when the time course of the heat curve was exponential.

Fluorescence measurements

The surface fluorescence of one of the muscles was excited at an angle of approximately 45°, the light passing through a window on the wall of the perfusion chamber, with a filter (Eppendorf 366) selecting the 366 nm line of a high stability spectral mercury lamp (Osram Hg/2). The signal emitted around 450 nm was collimated, sent through a secondary broad-band interference filter (Filtraflex Balzer K 2) coupled with a Balzer U.V. filter and collected on a photomultiplier (EMI, PM 9524 B). The photocurrent was recorded on the second beam of the galvanometer. The Kipp

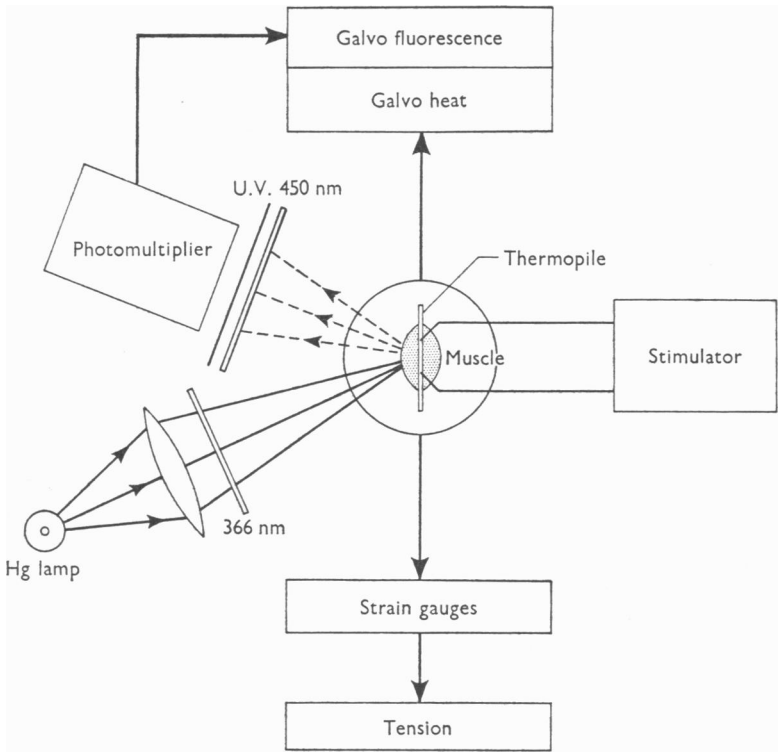

Fig. 1. Schematic representation of the experimental techniques.

Micrograph, exploring alternately each signal input, gave a discontinuous trace (10 dots/min, 1 min = 1 cm) while continuous records were obtained with the Sefram Graphirac. A schematic diagram is given in Fig. 1.

The fluorescence changes, also read every 0·5 min, were expressed with reference to the final base line for the kinetic determinations; in some cases, the fluorescence traces showed a more or less important displacement of the base line after completion of the fluorescence cycle (Fig. 5). This could be due partly to a movement of the muscles (it is indeed produced by stretching or shortening them) but partly also to some persistent metabolic variation.

Estimation of the parameters of a double exponential curve

The kinetics of both the aerobic recovery heat production and of the fluorescence changes could be satisfactorily described as the algebraic sum of two exponential terms:

$$Y_t = A e^{-at} \pm B e^{-bt}.$$

For the determination of the parameters of this curve, a 'peeling' method has been used, based on the areas delimited by each exponential. As explained under Results, the total area is indeed given by

$$\left(\frac{A}{a}\right) \pm \left(\frac{B}{b}\right);$$

it was approximated by numerical integration (Simpson formula). The individual areas have been used for the determination of the rate constants; the amplitude B of the slow component, was calculated by a least square method and A, the size of the fast component was obtained as the difference between the total measured amplitude and the size of B.

All these computations were performed on a Programma 101 Olivetti desk computer.

RESULTS

1. *The time course of the aerobic recovery heat*

It has already been suggested by A. V. Hill (1965) that the time course of heat production is approximately exponential during aerobic recovery. Fig. 2 confirms this point; when the heat production calculated in blocks of 0·5 min, ΔQ, is plotted in semilog scale against time, all the points after 4 min fall on a straight line. The inset shows that the first points can also be described in terms of another, faster, exponential curve. Thus the heat produced during recovery is described in good approximation by a double exponential curve of the form:

$$\Delta Q = A e^{-at} + B e^{-bt}. \tag{1}$$

It thus seems likely that the instantaneous rate of heat production has the same form

$$\frac{dQ}{dt} = \dot{Q} = \dot{Q}_A e^{-at} + \dot{Q}_B e^{-bt}, \tag{2}$$

where $\dot{Q}_A$ and $\dot{Q}_B$ are the maximum rates of heat production related to each component and a and b the corresponding rate constants.

The total recovery heat, Q_R, is represented by

$$Q_R = \int_0^\infty \dot{Q}\, dt = \frac{\dot{Q}_A}{a} + \frac{\dot{Q}_B}{b}, \tag{3}$$

where $\dot{Q}_A/a$ and $\dot{Q}_B/b$ symbolize the total heat produced by each component.

If the exponential model is adequate, Q_R, the calculated recovery heat, should be equal to the sum of the two components, if they were followed until the heat curve had returned to the base line. As the readings were usually limited to 10·5 min, the sum of ΔQ presents a small deficit of 2–6 % by comparison with Q_R (Table 2).

The ratio, R, of the total recovery heat, Q_R, to the initial heat, Q_I, will be the subject of another paper. It can vary substantially according to the

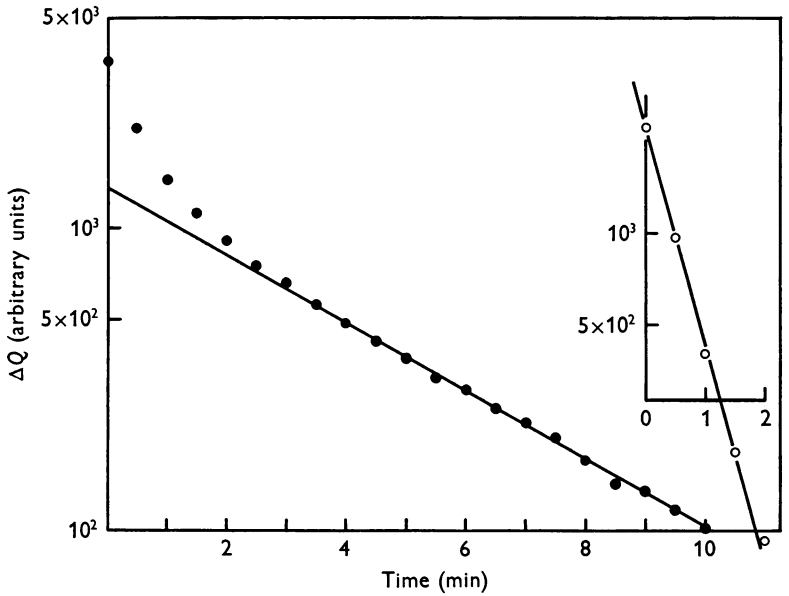

Fig. 2. Kinetic analysis of aerobic recovery heat production. In ordinate, the mean heat production calculated in blocks of 0·5 min interval in arbitrary units (log scale); in abscissa, the time in min. ●: experimental data and determination of the straight-line course of the slow term. In inset, the difference (○) between the experimental data and the corresponding values read on the extrapolated straight line, plotted on the same scales as the main Figure.

experimental conditions (see preliminary note, Godfraind-De Becker, 1971); it is especially high in winter toad muscles bathed in bicarbonate-Ringer.

Concerning the kinetics of the recovery process, it must be realized that we are not measuring the instantaneous rate of heat production, $\dot{Q}$, but the heat ΔQ produced during a constant interval Δt of 0·5 min. According to (2), the total heat produced up to the time t is equal to

$$Q_t = \int_0^t \dot{Q}\,dt = \frac{\dot{Q}_A}{a}(1-e^{-at}) + \frac{\dot{Q}_B}{b}(1-e^{-bt}) + C.$$

Therefore

$$\Delta Q = \frac{\dot{Q}_A}{a}(1-e^{-a\Delta t})e^{-at} + \frac{\dot{Q}_B}{b}(1-e^{-b\Delta t})e^{-bt}. \quad (4)$$

Comparing (1) and (4), it appears that the measured amplitudes A and B depend on the time interval used but it is easy to deduce from them the physiologically more interesting parameters $\dot{Q}_A$ and $\dot{Q}_B$. These are given,

TABLE 1. Kinetic parameters of the heat production during aerobic recovery after a tetanus of 0·5 sec at 20° C

	n	$\dot{Q}_A$	a	$\dot{Q}_B$	b
Normal (I)	5	14·7 ± 2·8	1·42 ± 0·15	5·8 ± 0·5	0·26 ± 0·01
(II)	5	16·5 ± 3·2	1·22 ± 0·09	7·3 ± 1·0	0·31 ± 0·04
IAA	3	12·7 ± 3·2	2·22 ± 0·40	7·7 ± 0·6	0·29 ± 0·03

The values reported are the means and the S.E. of mean. (I) and (II) refer to the first and second series of tetani on the same five muscles, three of which were later poisoned with 0·4 mM-IAA. $\dot{Q}_A$ and $\dot{Q}_B$ (mcal.g^{-1}.min^{-1}) represent the maximum rates of heat production connected with the fast and the slow components respectively, a and b (min^{-1}) are the corresponding rate constants.

TABLE 2. The total heat produced during and after a single tetanus of 0·5 sec in O_2 at 20° C

	n	Q_I	Q_A	Q_B	Q_R	Sum ΔQ	Sum $\Delta Q/Q_R$
Normal (I)	5	16·1 ± 0·9	10·1 ± 1·4	22·3 ± 1·9	32·4 ± 3·1	31·3 ± 2·9	0·96 ± 0·01
(II)	5	17·7 ± 0·7	13·4 ± 2·0	23·9 ± 2·2	37·3 ± 3·9	35·8 ± 3·9	0·96 ± 0·01
IAA	3	19·4 ± 0·8	5·8 ± 1·1	26·4 ± 1·2	32·2 ± 1·7	30·7 ± 1·4	0·95 ± 0·01

The values reported are the mean and the S.E. of mean. The heats are expressed in mcal.g^{-1}. Q_I represents the initial heat, Q_A and Q_B the recovery heat related to the fast and slow components respectively of the total recovery heat, Q_R. The recovery heat measured up to 10·5 min is given under Sum ΔQ. The ratio between the measured and the calculated heat is given in the last column.

together with the rate constants a and b in the two first lines of Table 1 corresponding respectively to the first and second series of normal contractions.

As can be seen, the results are fairly consistent. In particular, the variations between the two series are small and quite insignificant.

After poisoning of the muscles with 0·4 mM-IAA, the recovery heat can still be described in terms of two exponentials; the parameters, given in the third line of Table 1, are hardly different from the normal ones, except for the rate constant of the fast component, a, which was significantly increased by IAA ($0·01 < P < 0·02$). There is also an indication that the size of $\dot{Q}_A$ is slightly depressed; though the difference is not significant by the usual t test, it appears real when the comparison is made for each individual

muscle. Altogether, the total heat Q_A ($= \dot{Q}_A/a$; see eqn. (3)), connected with the fast exponential term is markedly reduced, from 13·4 mcal.g^{-1} immediately before to 5·8 after intoxication (Table 2). On the other hand, the slow component Q_B of the recovery heat is increased, so that on the whole the total recovery heat is hardly changed by IAA.

2. *The fluorescence cycle following a tetanus: general description*

(*a*) *Normal aerobic muscle*

Fig. 3 shows the succession of fluorescence cycles during one experimental series. Although, as far as the absolute magnitudes are concerned, the fluorescence cycle is more variable than the heat production, the sequence of events is rather consistent. After a tetanus of 0·5 sec at 20° C, the sequence is as follows. During contraction, there is a very rapid change of

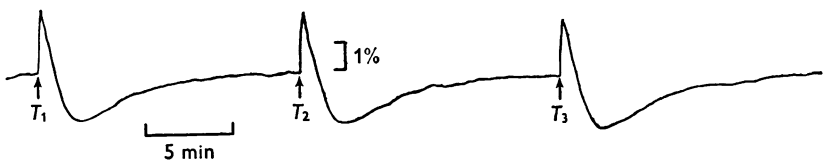

Fig. 3. The fluorescence changes evoked by three successive tetani of 0·5 sec at 20° C in normal muscle. A fluorescence increase corresponds to an upwards deflexion. T_{1-2-3} indicate the successive tetani. The deflexion simultaneous with the contraction is not recorded (Kipp's discontinuous trace).

the emitted light, the time course of which was obscured by a movement artifact. In most examples given, recorded with the discontinuous trace system (Kipp's galvanometer), this artifact was dropped out. The example of Fig. 4*a* shows a continuous trace (Sefram Graphirac) where it is present. In all but one of the experiments where it could be observed, the net result was a decrease of the fluorescence intensity. This initial change is rapidly reversed and is succeeded by an increase reaching a maximum after 10 to 15 sec. Then follows a progressive diminution of fluorescence, with a second crossing of the base line, the minimum being attained in 1·5 to 2·5 min. The cycle is completed by a slow increase of the emitted light; in most cases the fluorescence trace eventually stabilizes at or near the initial level.

The magnitude of the maximum increase of fluorescence, after relaxation, is between 0·5 and 1·5% of the basal intensity. The minimum of fluorescence, observed later, corresponds to a change between 1 and 3% from the initial base line.

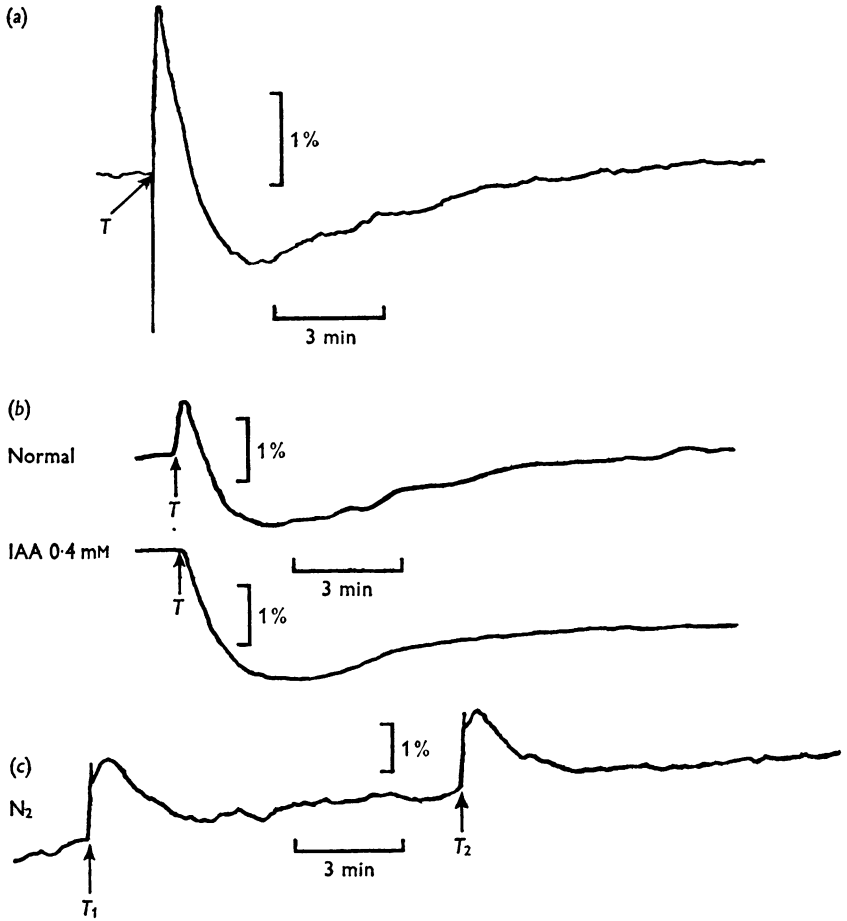

Fig. 4. The fluorescence changes evoked by a tetanus of 0·5 sec at 20° C in various conditions.

(*a*) The uppermost trace shows a continuous record of fluorescence under normal conditions with the movement artifact during the contraction.

(*b*) The two following traces represent the fluorescent changes observed on the same muscle in normal conditions and after IAA poisoning. *T* indicates the time when the tetanus was elicited.

(*c*) The lower trace shows the variations evoked by two successive tetani in nitrogen (T_1, T_2).

An increase of fluorescence corresponds to an upwards deflexion.

(*b*) *IAA poisoned aerobic muscle*

After intoxication by IAA, the very rapid initial change during the contraction persists; but after a fast return towards the base line the fluorescence shows only a decrease of intensity followed by an increase. The amplitude of this diminution is approximately 2–3% of the basal

intensity. The most obvious effect of IAA, from a qualitative point of view, is thus the suppression of the fast increase of fluorescence initiating the recovery cycle in normal muscle (Fig. 4*b*). At the beginning of intoxication, the trace may return to the base line just as before poisoning but with the progress of intoxication this return becomes more and more incomplete (Fig. 5) until the trace stabilizes at a minimum level of fluorescence.

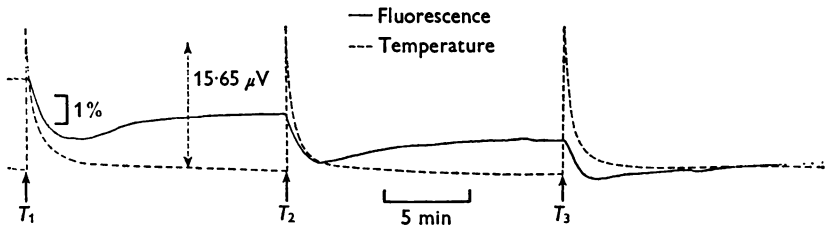

Fig. 5. Evolution of fluorescence and temperature traces evoked by successive tetani after IAA poisoning. Increases of temperature (– – –) and fluorescence (——) correspond to upwards deflexions. T_1,T_2 and T_3 indicate the successive tetani; the movement artifacts are not recorded. Note the progressive fall of the fluorescence signal, while the initial heat deflexion, if anything, increases slightly from tetanus to tetanus.

(*c*) *Anaerobic muscle*

In the course of a few experiments, the muscles were submitted to anaerobiosis, either in the gas phase or in the Ringer fluid, by changing the gas mixture for N_2–5% CO_2. Such a transition is followed by a large increase of the basal fluorescence intensity (Lebacq & Aubert, 1969). After 30–45 min, tetanic stimulation produced almost normal tension and initial heat but evoked only a rather fast initial increase of fluorescence followed by a return to the base line (Fig. 4*c*). The amplitude of the variation was about 1% of the initial intensity.

3. *The time course of the fluorescence cycle*

If the initial step of fluorescence increase during the 10–15 sec after contraction is ignored, the main course of the fluorescence pattern in normal muscle is suggestive of a double exponential function with the two components in opposite directions. By analogy with the kinetics of recovery heat, the fluorescence cycle has been analysed according to the mathematical model specified by the equation:

$$F = A'e^{-a't} - B'e^{-b't}.$$

Such a description reasonably fits the experimental data read every 0·5 min during 10·5 min, when the first rapid changes are neglected. A similar analysis is applicable for the IAA-poisoned muscle.

The rate constants, in min^{-1}, and the amplitudes of each component, in nA of photocurrent, are given in Table 3, which corresponds to Table 1 for the recovery heat.

It is seen that IAA produces an acceleration of the time course of the fast component, its rate constant a' being significantly increased ($P < 0{\cdot}01$). The slow phase seems not to be affected.

TABLE 3. Kinetic parameters of the fluorescence cycle following a tetanus of 0·5 sec in O_2 at 20° C

	n	A'	a'	B'	b'
Normal (I)	5	71 ± 19	$0{\cdot}80 \pm 0{\cdot}04$	59 ± 17	$0{\cdot}24 \pm 0{\cdot}03$
(II)	5	79 ± 13	$0{\cdot}88 \pm 0{\cdot}04$	50 ± 6	$0{\cdot}28 \pm 0{\cdot}04$
IAA	3	67 ± 31	$1{\cdot}31 \pm 0{\cdot}08$	53 ± 23	$0{\cdot}30 \pm 0{\cdot}05$

The values reported are the mean and S.E. of mean. A' and B' represent the absolute amplitude (in nA of photocurrent) of the fast and slow components respectively; a' and b' are the corresponding rate constants (min^{-1}).

DISCUSSION

1. *The aerobic recovery heat production*

A.V. Hill (1965, p. 195) has already noted that in frog sartorius at 0° C, from 3 min after the contraction onwards, the aerobic recovery heat has an exponential time course with a rate constant of 0·1 min^{-1}. He observed a similar exponential evolution in toad sartorius at 20° C from 0·5 min after a tetanus lasting 3·2 sec, with a rate constant about 0·5 min^{-1}. In our experiments, this simple evolution appears only later, and most of the kinetics of the aerobic recovery heat production is best described in terms of the sum of two decreasing exponential components. In toad sartorii buffered by bicarbonate-CO_2, after a tetanus of 0·5 sec at 20° C, the rate constants of the two components are respectively about 1·3 min^{-1} and 0·3 min^{-1}. The difference from Hill's results may arise from various factors (e.g. duration of stimulation, buffer, temperature), but the most important is probably the time during which the heat production is followed. In the example published by Hill, the recovery heat was analysed during only 4 min. It is probable that an estimate of kinetics on such a short time period may be biased, especially if two exponential terms are superimposed with a ratio of only three to four between their rate constants.

Concerning the effects of iodoacetate, it has long been known (Cattell, Feng, Hartree, Hill & Parkinson, 1931) that the first contractions after poisoning are followed by an apparently normal recovery heat. As far as the total recovery heat is concerned, our experiments confirm this finding (Table 2). It is only when the effects of intoxication develop further with

a fall of tension and of initial heat, that the recovery heat is seriously affected, giving a significant decrease of the recovery ratio (recovery heat/initial heat). On the other hand our experiments show that the time course of the recovery heat is modified by IAA from the start. According to Table 1, the rate constant of the rapid component shows a large increase from 1·22 to 2·22 min^{-1} though that of the slow component is hardly changed. It thus seems clear that already in its early phase IAA-poisoning induces modifications of the kinetics of recovery. There is also a change in the ratio of the amounts of heat produced in the two phases, though not in their total (Table 2).

Finally, it should be mentioned that the results of a few experiments with muscles under anaerobic conditions indicate that the recovery heat production is greatly shortened in duration and may be described by a single, relatively fast, exponential with a rate constant between 0·6 and 1·7 min^{-1}. This confirms previous observations on the frog sartorius (Hill, 1965; p. 175). Taken together, the effects of IAA and of anaerobiosis show clearly that the slow exponential component has a purely aerobic origin, as the discussion of fluorescence will confirm.

2. *The fluorescence cycle following a short tetanus*

It is clear that the observations here described differ from those of Jöbsis (1963) and Jöbsis & Duffield (1967) who mostly observed a cycle of decreased fluorescence after activity. The dissimilarity cannot be assigned to the movement artifact: it is indeed long over when the post-contractile fluorescence changes begin. But the species difference and the mode of excitation may well account for it. Jöbsis & Duffield (1967) studied the fluorescence changes at lower temperature after a series of twitches on aged sartorii of the toad *B. marinus*. At room temperature and on freshly excised muscles, Jöbsis (1963) himself had noted that the fluorescence cycle might become biphasic and that the tendency towards a reduction increased with the amount of contractile activity. On freshly excised muscles of *B. marinus* we could observe, as Jöbsis (1963) did, that a series of twitches evoked a decrease of fluorescence which was reversed and followed by an increase of the emitted light before returning to the resting state.

It is generally accepted that, when a tissue is illuminated with ultraviolet light around 366 nm, the fluorescence emission around 450 nm provides an index of the oxidation-reduction state of the nicotinamide-adenine dinucleotides (Chance, Jöbsis, Cohen & Schoener, 1962). NADH is fluorescent and NAD^+ is not, so that a decrease of the intensity of the light emitted corresponds to a more oxidized state of the coenzymes, while an increase corresponds to a more reduced state. Interpreting our observations

along these lines, it appears that a brief tetanus of a normal muscle in oxygen evokes a complex response. Following the change during contraction, which is confused by the movement artifact, the post-relaxation fluorescence cycle is initiated by a rapid transition to a more reduced state. This initial step is followed by an oxidation of NADH which attains a maximum and then slowly reverses, with a return to the resting state.

If this cycle presents a specific picture of the activity of the respiratory chain, as has been suggested by Jöbsis (1963) and with some reservations by Jöbsis & Duffield (1967), an inhibitor of glycolysis such as IAA should not modify it significantly. Our results show that, on the contrary, IAA produces qualitative as well as quantitative changes: the initial step of reduction of the coenzymes is totally suppressed and the rate constant of the fast phase (decrease of fluorescence) is significantly increased. It thus appears that even in oxygenated toad muscles, rich as they are in mitochondria, glycolytic reactions can influence the course of fluorescence.

Such an influence of glycolysis has already been observed in other organs (Williamson & Jamieson, 1965; Brauser, Bücher & Dolivo, 1970; Kohen, Kohen, Thorell & Åkerman, 1968; Aubert, Chance & Keynes, 1964). The initial increase in fluorescence seen in the normal muscle could reflect either a direct effect of glycolysis (reduction of the cytoplasmic coenzymes at the step of the glyceraldehyde-3-phosphate dehydrogenase) or an indirect effect (reduction of the mitochondrial couple as a result of the metabolism through the tricarboxylic acid pathway of some substrate formed by glycolysis or as a decrease of the ADP level). Jöbsis & Duffield (1967) favour the second hypothesis to account for the oscillations of fluorescence they observed under some conditions; though it is clear that in the experiments of Kohen *et al.* (1968) and Aubert *et al.* (1964) direct effects must have been responsible.

The best argument in favour of a direct effect of glycolysis in our experiments is the fact that when a muscle contracts in nitrogen it still shows fluorescence changes. It is restricted then to the fast initial reduction of NAD^+ (Fig. 4*c* and Godfraind-De Becker, 1968) followed by a return to the base line. On the other hand, the long lasting phase of decreased fluorescence is most probably related to mitochondrial events as it disappears in nitrogen but persists largely unchanged in IAA (Fig. 4*b* and Table 2).

It may thus be concluded that the fluorescence cycle represents a complex pattern of metabolic processes just as does the heat production. Even if it may be interpreted in terms of a single event (the reversible transformation of NADH into NAD^+) it is probable that pyridine nucleotides at more than one location are involved. The bound mitochondrial NADH normally account for the greatest part of the light emission, but

our results show that the role of the free cytoplasmic reduced coenzymes cannot be neglected.

The following reactions can thus be invoked to explain the observed fluorescence changes:

(1) the activity of the glyceraldehyde-3-phosphate dehydrogenase, increasing rapidly the NADH level; this reaction would account for the initial step of increased fluorescence. It is specifically inhibited by iodoacetate, which has been shown to suppress this early change;

(2) the activity of the cytochrome system, responsible for a rapid but slightly delayed oxidation of the mitochondrial NADH; it would account for the decrease of light emission corresponding to the fast phase of the fluorescence cycle;

(3) the activity of the tricarboxylic acid cycle, producing NADH at a slower rate; it would account for the slow phase of the fluorescence change. The relation of the slow phase with the Krebs cycle could be tested by the use of a specific inhibitor. Nevertheless there is an indication that favours this hypothesis: the progressive impairment of the final reduction in the IAA-poisoned muscle could be ascribed to a progressive decline of activity of the citric acid cycle due to a gradual lack of substrate.

3. *The correlation between recovery heat and the fluorescence changes*

Table 4 presents in parallel form the kinetics of the two monitors analysed respectively in Tables 1 and 3. It shows the similarity of the time course of the slow components for both heat and fluorescence, which contrasts with the different kinetics of the fast components.

When the 'slow' rate constants b and b' are compared by the paired t test for the same muscles within each series, it is found that the very small differences between the means are completely insignificant either in the normal ($P = 0{\cdot}6$) or in the IAA-treated muscles ($P = 0{\cdot}9$). The same test shows that the large difference found between a and a' is quite significant ($P = 0{\cdot}01$) and is made still larger by IAA, which increases both of them ($P = 0{\cdot}15$).

It is thus clear that in the second phase of recovery, i.e. 4 or 5 min after the tetanus, heat and fluorescence monitor the same metabolic events, at a time when, as already shown by D. K. Hill (1940*a*), they are purely aerobic. In both cases the slow component is hardly affected by IAA but disappears in nitrogen. The facts are more complex during the first phase of recovery. Not only are the rate constants of the first exponential phase different for heat and fluorescence, but it seems impossible to ascribe it to a single process such as glycolysis. It is true that, as suggested above, glycolysis may be playing a part during early recovery, even after a short tetanus; but our results indicate that the fast exponential is related to

oxidative and glycolytic processes occurring side by side, for they are not suppressed but only modified by IAA, while they persist in nitrogen.

As for the difference between the two 'fast' rate constants, it could have a double origin: first, heat and fluorescence are not monitoring exactly the same process, the heat measuring the global intensity of all chemical and physical processes while fluorescence specifically reflects the oxidation-reduction state of the pyridine nucleotide couple. Secondly, even if there was a complete parallelism between the time course of heat and fluorescence, our analysis postulating a double exponential model could be distorted if a third and still faster component exerted its effects in the earliest

TABLE 4. Time course of the aerobic recovery measured by the heat rate ($\dot{Q}$) and the fluorescence pattern (F) after a tetanus of 0·5 sec at 20° C

	$\dot{Q}$	F
Normal (I)	$14{\cdot}7\ e^{-1{\cdot}42t} + 5{\cdot}8\ e^{-0{\cdot}26t}$	$71\ e^{-0{\cdot}80t} - 59\ e^{-0{\cdot}24t}$
(II)	$16{\cdot}5\ e^{-1{\cdot}22t} + 7{\cdot}3\ e^{-0{\cdot}31t}$	$79\ e^{-0{\cdot}88t} - 50\ e^{-0{\cdot}28t}$
IAA	$12{\cdot}7\ e^{-2{\cdot}22t} + 7{\cdot}7\ e^{-0{\cdot}29t}$	$67\ e^{-1{\cdot}31t} - 53\ e^{-0{\cdot}30t}$

All the parameters are expressed in the same units as in Tables 1 and 3.

stages of recovery. According to numerical tests, the presence of a third exponential factor with a reverse direction for both phenomena could well produce differences such as 0·8 and 1·4 seen in Table 4. Such a possibility is not illusory even though any distortion of the recovery processes by the slowness of the oxygen diffusion has been prevented by the use of tetani of short duration. A. V. Hill (1961) has shown that after a short tetanus there is an early phase of heat absorption, rapidly reversed in oxygen at high temperature. Though under the conditions of analysis here used, only positive heat production was observed during the first $\frac{1}{2}$ min interval, the rapid sequence of events described by Hill (1961) may well have influenced the estimation of the rate constant of the first exponential. On the other hand, the initial oscillation of the fluorescence trace might also favour such an interpretation. The effect of IAA is interesting to note in this context: IAA suppresses the initial peak of reduction in the fluorescence trace but does not inhibit the negative delayed recovery heat (D.K. Hill, 1940*b*; A. V. Hill, 1961); and Table 4 shows that, if anything, IAA reinforces the discordance between the two 'fast' rate constants.

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