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Rapid microfluorimetry of enzyme reactions in single living cells

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SUMMARY

An optimized photon counting technique allows the microfluorimetric study of NAD^+ (or $NADP^+$) reduction–reoxidation transients in single living cells with a time resolution in the range of 1/50–1/100 sec. The transients resulting from the microelectrophoretic addition of metabolites (*e.g.* Glc-6-*P* or Glc-1-*P*) can be analyzed in terms of early parameters (*e.g.* initial lag, rise half time or full rise time) and overall parameters (time of rise and half decay, amplitude, reoxidation time). Both the initial lag and rise half time are considerably longer with Glc-1-*P* than with Glc-6-*P*, possibly due to control at the phosphoglucomutase or compartmentation of glycolytic phosphate esters. While glycolytic NAD^+ (or $NADP^+$) reduction proceeds adequately in aerobic EL2 and EAT ascites cells (although $\Delta NADH/\Delta t$ is higher at anaerobiosis), it is critically dependent upon anaerobiosis in L and astrocytoma cells. Thus by rapid microfluorimetry it is possible to resolve the rising phase or other segments of the fluorescence transients into components each corresponding to a particular step in the sequence of intracellular events or control states.

NAD^+ or $NADP^+$ reduction–reoxidation transients resulting from the microelectrophoretic addition of substrates, *e.g.* glucose 6-phosphate (Glc-6-*P*) to the cytoplasm or nucleus of single living cells can be detected in a modified Chance-Legallais microfluorimeter with an overall response time of around 1 sec[1,2]. The fluorescence pulses[3] which correspond to these transients provide a preliminary basis for the evaluation of substrate conversion rates, NADH accumulation as well as fractional reduction of NAD^+. However, in the fluorescence pulse there are components such as the rapidly rising initial portion (under the most simplified assumptions a measure of glyceraldehyde phosphate dehydrogenase activity when glycolytic substrate is added) which escape detailed analysis as they correspond to events lasting only a few 100 msec. The fluorescence excitation and optical arrangements for these experiments have been described elsewhere[2].

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Photomultiplier detection of fluorescence is limited by the formula[4]:

$$\text{Noise} = \sqrt{2 \times \text{emitted photoelectrons} \times \text{bandwidth of measuring equipment in sec}^{-1}}$$

When (as in these experiments) incident photons arising from cellular components lead to a photocathode emission at the rate of 20 000–40 000 electrons per sec, the best signal-to-noise ratio that is obtainable with a time resolution of 1/50 sec falls in the range of 20 (or 30) to 1. For each photon annihilation at the cathode, a pulse (10^5–10^6 electrons) lasting a few nsec is observed at the anode, the multiplication process depending on a statistical distribution of secondary emission probabilities. The time t taken to achieve a desired accuracy is dependent on the storage technique (*e.g.* digital *vs.* capacitive) and standardization of photomultiplier output. While the respective merits of the photon counting[5–8] *vs.* condenser integrating[1,7,8] techniques have led to some controversy[6–8] (shorter t claimed for the former), on the basis of actual performance an optimized photon counting system[9,10] brought to the limits of theory has been found suitable for rapid microfluorimetry (Fig. 1). Long-term stability is achieved by operating an EM1 9502 SA photomultiplier in a "plateau region" with a count rate first-order independent of high

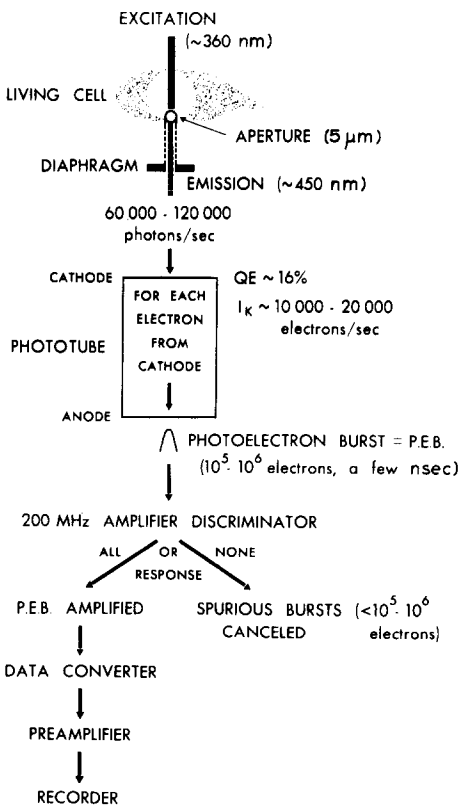

Fig. 1. Schematic representation of the photon counting technique used for rapid microfluorimetry. QE = quantum efficiency.

voltage. Base-line wanderings from tube-leakage currents are eliminated by the low-frequency cutoff of an SSR Instruments Co. Model 1120, 200 MHz amplifier discriminator (gain, 2400; threshold, 25 μV; nominal dead time, 25 nsec). The dead time in between measurements can improve the fluorescence to background ratio if the former is more randomized in time (*i.e.* as coincident photons were observed in mercury are emissions[5]). Spurious electron pulses originating at various stages along the multiplication chain are eliminated by the discriminator threshold. Standardized photoelectron pulses receive equal weighting in a digital to analog converter (SSR Instruments Co. Model 1105 data converter) to yield d.c. signals continuously displayed on a front panel and made available for a Hewlett Packard recorder.

Among the factors which can influence the slope ΔNADH (or NADPH)/Δt or (Δ fluorescence intensity)/Δt of the rapidly rising fluorescence pulse (*e.g.* when Glc-6-*P* is added) there are: physicochemical processes (*e.g.* diffusion and dynamic organizational or structural changes at the submicroscopic level), experimental circumstances (*e.g.* ion population at the micropipette tip), the built-in biochemical state of the cell and the relative paces or interdependence of various multienzyme pathways involved in the utilization of substrate. In an average EL2 or EAT ascites tissue culture cell, the distance (d) between the micropipette tip and the site of observation will be such that under the assumption of free diffusion[11] (*plus* electromigration[10,12]) the ejected ions should have crossed d in no longer than 20–30 msec. The rapid build-up of required substrate concentrations can be managed by regulating the microelectrophoretic voltage and by means of an interval timer allowing ejection times as short as 20–50 msec. The actual mixing half time is found to be 80 msec (Table I, footnote★) when fluorescein is added micro-

TABLE I

EARLY PARAMETERS OF FLUORESCENCE RISE WITH INTERMEDIATES OF GLUCOSE METABOLISM

Most experiments were performed in aerobic preparations of EL2 cells (except for UDPG, in anaerobic L cells). Anaerobiosis was required for detectable fluorescence changes with fructose 1,6-diphosphate (Fru-1,6-P_2) and glyceraldehyde phosphate (glyceraldehyde-*P*) (*plus* incubation with 6.1 mM ethionine to lower ATP[13]). Lag★ = delay from the start of microelectrophoresis to onset of fluorescence rise; $t_{1/2on}$★ = rise half time. S.E. = $\pm\sqrt{\Sigma d^2/n(n-1)}$.

Metabolite	*Lag*	$t_{1/2on}$
Glc-1-*P*	700 ± 130	3060 ± 330
Glc-6-*P*	250 ± 50	1100 ± 60
Fru-6-*P*	110 ± 40	940 ± 110
Fru-1,6-P_2	160 ± 70	1000 ± 220
Glyceraldehyde-*P*★★	50	1000
6-Phosphogluconate	350 ± 110	1440 ± 250
UDPG★★★	800 ± 160	1270 ± 130

★In similar experiments with fluorescein there was no lag, $t_{1/2on} = 80 \pm 15$.
★★Single determination, due to the difficulty in displacing the NAD^+–NADH equilibrium with glyceraldehyde-*P* alone.
★★★In presence of UDP, to minimize channeling towards glycogen.

electrophoretically within 50 msec. When the homeostasis of the living cell is challenged by the superimposition of a metabolic transient, there occurs a change in the redox state of the hydrogen carriers (Fig. 2). The observed redox change is a composite of the over-all metabolic capacity expressed by cofactor availability, efficiency at points of metabolic control, allosteric state of some enzymes, *etc.*

The first analysis of a biological system by rapid microfluorimetry has consisted in a reevaluation of glucose catabolism along various pathways directly *in situ*, within the extramitochondrial compartment of single living EL2 or L cells (Table I). With Glc-6-*P* or fructose 6-phosphate as the substrate, the initiation of glycolytic NAD^+ reduction may be preceded by a 100–200-msec lag (Table I, Fig. 2). This lag is prolonged considerably (Fig. 3) when glucose 1-phosphate (Glc-1-*P*) is substituted for Glc-6-*P* (700 ± 130 msec (n = 15) *vs.* 245 ± 45 msec (n = 11)), possibly due to control at the phosphoglucomutase step[13,14] or to intracellular compartmentation of glycolytic phosphate esters[15] (*e.g.* multiple pools[15], with Glc-6-*P* derived from Glc-1-*P* not necessarily distributed like Glc-6-*P* added by micro-electrophoresis).

The slope of the fluorescence rise ($\Delta NADH/\Delta t$) can be evaluated as the rate of change in photon counts, or photon counts per sec/Δt sec (= photons/sec^2). In an idealized model $\Delta NADH/\Delta t$ should exhibit a constant relationship to substrate concentration[16]. The cell can cope with small concentrations of Glc-6-*P* (*e.g.* 10 μM) with no displacements in the NAD^+–NADH equilibrium[3]. The steady-state level of glycolytic NAD^+ or $NADP^+$ reduction can be displaced out of equilibrium by further addition of Glc-6-*P* or fructose 6-phosphate to the cytoplasm (or nucleus) of EL2 cells[3]. At physiological levels (*e.g.* 100 μM to 1 mM) $\Delta NADH/\Delta t$ is roughly proportional to substrate concentration but it tends to level off and decrease beyond a certain substrate threshold.

There are preliminary indications that for a comparable substrate level $\Delta NADH/\Delta t$ can be made to vary by altering the metabolite mixture or conditions of cells incubation. With Glc-6-*P* it amounts to approx. 3000–4000 photons/sec^2 at aerobiosis *vs.* 8000 at anaerobiosis and 10 000–20 000 in presence of lactate. The rise half time ($t_{1/2\,on}$) of the fluorescence curve with Glc-6-*P* varies from 200 to 2000 msec. Between $t_{1/2\,on}$ and full rise time, there is often a sudden change in slope with slowing of $\Delta NADH/\Delta t$ from 1/4 to 1/10 of the original rate. Thus the rising branch of the fluorescence curve can exhibit a triphasic appearance: lag–rapid rise–slow rise. The slow and terminal portion of the rising branch is suggestive of metabolic control which possibly sets in once a certain threshold of substrates utilization or co-enzyme reduction is exceeded.

In EL2 and EAT ascites cell cultures, the rates of Glc-6-*P* or Glc-1-*P* utilization (as calculated from the transient half time) vary from 60 to 500 μmoles $\cdot$ sec^{-1} $\cdot$ kg^{-1}, possibly due to endogenous levels and/or external additions of cofactors[3] (*e.g.* adenine nucleotides). While glycolytic reduction of NAD^+ or $NADP^+$ can be observed adequately in EL2 cells maintained under aerobic conditions, in cultures of L cells and human astrocytoma, this reduction is critically dependent upon anaerobiosis, the addition of NAD^+, or that of mitochondrial inhibitors and ionophores (*e.g.* valinomycin[17]). In presence of valinomycin, $\Delta NADH/\Delta t$ is increased by 3 to 4 times, while Glc-6-*P* utilization increases from 10 to 150 μmoles $\cdot$ sec^{-1} $\cdot$ kg^{-1}.

Thus, by rapid microfluorimetry it is possible to resolve the rising phase of the fluorescence pulse into components each corresponding to a characteristic phase in the

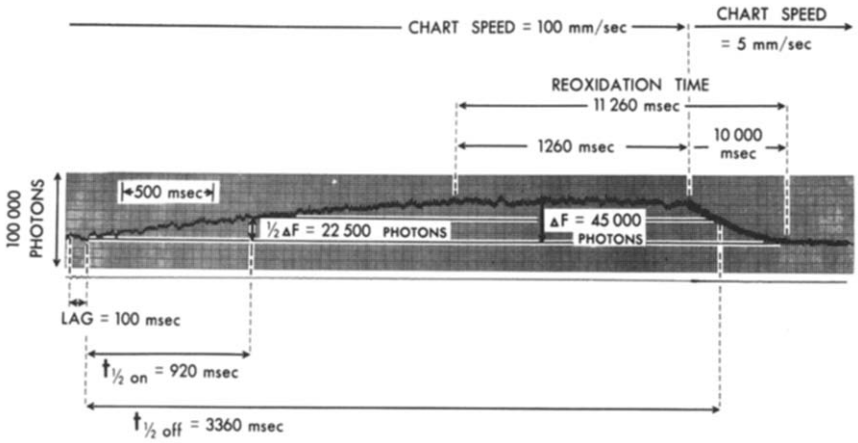

Fig. 2. Rapid recording of a microfluorimetric pulse (NAD^+ reduction–reoxidation transient) from the extramitochondrial space of an L cell following microelectrophoretic addition of Glc-6-*P*. The marker trace ~~indicates the time and duration of the microelectrophoretic addition. ΔF= amplitude of the fluorescence rise, $t_{1/2on}$ =rise half time, $t_{1/2off}$= half time of rise and decay. While reoxidation time is started for convenience from the termination of full rise time, it is likely that reoxidative processes start much earlier (as possibly suggested by a change in slope around $t_{1/2on}$). Cell MA4-71-L/III-H.

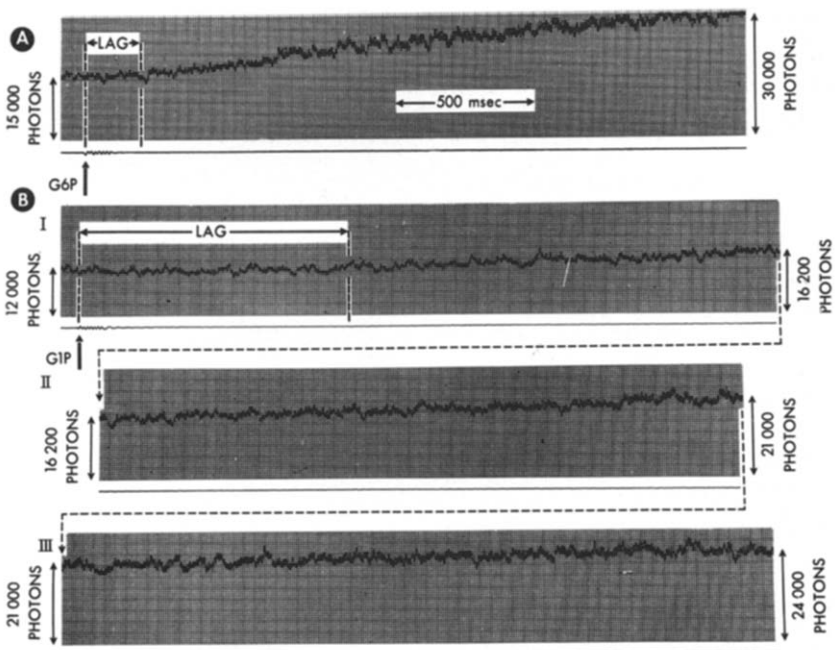

Fig. 3. Lags preceding the onset of the NAD^+ reduction–reoxidation transients: (A) with Glc-6-*P* (G6P), (B) with Glc-1-*P* (G1P). Both microfluorimetric curves were recorded from the extramitochondrial space fo EL2 cells. The very slow rise with Glc-1-*P* is seen in frames I, II and III. Cells FB3-71-EL2/I-4 and FB3-71-EL2/IV-5.

sequence of intracellular events or control states. The time resolution is also more in agreement with the actual velocities at which multi-enzyme reactions proceed *in situ*, and also more at pace with other metabolic or structural parameters (*i.e.* metabolic lags (see Glc-1-*P* effect) or delays due to membrane barriers (*e.g.* mitochondrial)).

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