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ENZYME–SUBSTRATE KINETICS ASSOCIATED WITH TRANSIENT STATE CONDITIONS IN SINGLE LIVING CELLS

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SUMMARY

1 The transient changes in NAD^+ reduction (fluorescence pulse) which result from the microelectrophoretic addition of substrate in stepwise increasing amounts can be followed in single living EL2 cells by microfluorimetry With glucose 6-phosphate (Glc-6-*P*) as substrate, each fluorescence pulse represents the summated contributions of glyceraldehyde phosphate dehydrogenase (NAD^+-reducing) and lactic dehydrogenase (NADH-reoxidizing)

2 The plot of Glc-6-*P* concentration against the area of the fluorescence pulse reveals sigmoidal kinetics, which are altered by adenine nucleotides, lactic dehydrogenase inhibitor and substrates, *etc* In all these cases the kinetics of NAD^+ reduction *in vivo* favour a higher-order relationship with regard to substrate in the optimum concentration range

3 When Glc-6-*P* is increased gradually metabolic block sets in more easily than if optimal doses are added at once or suboptimal doses by continuous slow drip

4 While the substrate levels required for half-maximal and maximal NAD^+ reduction are somewhat lowered in presence of ADP and P_i as compared to Glc-6-*P* alone, there is considerable parallelism between the rates of substrate utilization under all these conditions The rate is severely reduced in presence of ATP and maximal NAD^+ reduction occurs at a much lower concentration of Glc-6-*P*

5 At suboptimal or optimal levels of substrate, lactic dehydrogenase is quite efficient in reoxidizing NADH, but the latter can accumulate considerably when the cell is overloaded with substrate In presence of lactate and oxamate the rates of Glc-6-*P* utilization are considerably lowered, but the NAD^+ reduction proceeds quite adequately and occurs at substrate concentrations much lower than with Glc-6-*P* alone

6 The EL2 cells seem self-sufficient in terms of endogenous activators of the glycolytic chain, so that they depend mainly on the supply of substrate However, in the living cell the interplay between mitochondrial and extramitochondrial compartments is suggested by succinate and ATP experiments

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INTRODUCTION

Since the development of the beam-splitter supplemented Chance–Legallais microfluorimeter[1–3] it has been possible to follow continuously in a single living cell the transient fluorescence changes associated with pyridine nucleotide reduction upon microelectrophoretic additions[4] of substrates. If glycolytic substrates metabolizable by NAD^+ or NADP-linked enzymes or their precursors (*e g*, glucose 6-phosphate (Glc-6-*P*)) are added, a fluorescence pulse is observed which represents under the most simplified conditions the algebraic sum of forward and backward reactions at the glyceraldehyde phosphate dehydrogenase (NAD^+-reducing) and lactic dehydrogenase (NADH-reoxidizing).

The first illustrative examples of the technique[3,5,6] were obtained by adding glycolytic intermediates in stepwise increasing amounts to the cytoplasm of ascites cells in culture (EL2 cells) and following the fluorescence changes in the extramitochondrial compartment (cytoplasmic or nuclear region). Preliminary experiments[4] with a glycolytic intermediate (Glc-6-*P*) have shown that a sigmoid curve is observed when the areas of successive fluorescence pulses are plotted against concentration, an observation which is not inconsistent with the fact that most regulatory enzymes are known to catalyze reactions kinetically of second or higher order with respect to substrates and regulatory metabolites[7,8].

Although there could be other interpretations (*e g*, relaxation effects[7] or endogenous substrate thresholds) the above sigmoidal kinetics recall the properties of allosteric enzymes[7,8]. Therefore it is of interest to determine to what extent such kinetics are affected by agents known to control glycolysis in ascites cells (*e g*, adenine nucleotides, P_i, *etc*.). In fact the kinetics are found to respond quite sensitively to the rate, rhythm and sequence of substrate administration, as well as to the simultaneous presence of activators or inhibitors.

MATERIALS AND METHODS

The beam-splitter supplemented Chance–Legallais microfluorimeter[1–4] for the kinetic study of fluorescence changes synchronously with cell manipulations, the EL2 cells[9–12] used in these experiments and the microelectrophoretic technique[10–13] for the intracellular additions of substrates have been described previously.

Generally three modes of substrate administration were used on each experimental series, after determining the threshold level at which a barely detectable fluorescence response is recorded. (1) Substrate was consecutively administered to the same cell in stepwise increasing amounts, starting from a microelectrophoretic current of $1 \cdot 10^{-11}$ A and progressing beyond the level at which a maximum fluorescence is observed (dose-response curve). All currents were of 1-sec duration. (2) The above dose-response curve permits the localization of the intermediate concentration range (optimum) at which a rapidly increasing (from submaximal to maximal) fluorescence response is observed. By going over the optimum no further increase in pulse amplitude is recorded, but the pulse area can continue to increase due to broadening of the fluorescence pulse. Thus, a second mode of substrate administration consists of treating the cell directly with a maximal or submaximal dose of substrate at once. (3) A third method consists of applying continuously for 30 sec a microelectrophoretic

current below the optimum region, such as around $1\ 10^{-10}$ A (continuous delivery)

For these experiments Glc-6-*P* was used first alone and then in combination with adenine nucleotides, as well as other substrates or inhibitors The rationale for using activators or inhibitors of glycolysis in the same micropipette has been described previously[11,12,14] The micropipette was generally filled with a 2 M solution of Glc-6-*P* In glycolytic combinations Glc-6-*P*/adenine nucleotides = 8, Glc-6-*P*/P_i = 2, Glc-6-*P*/oxamate = 1 to 10, Glc-6-*P*/succinate = 1, Glc-6-*P*/lactate = 3

The peak amplitude of the fluorescence pulse obtained in response to Glc-6-*P* is evaluated in each case as a percent increase over the initial fluorescence prior to substrate addition It provides a measure of NAD^+ reduction

The initial concentration of substrate (*e g* , Glc-6-*P*) reached in the EL2 cell corresponds to the microelectrophoretically ejected amount in mole per cell volume in l For a 1-sec application of a microelectrophoretic current of $1\ 10^{-8}$ A, the number of elementary charges displaced should be roughly of $6\ 10^{18}$ (number of electrons per A) $10^{-8} = 6\ 10^{10}$ Dividing by the Avogadro number and the number of charges per Glc-6-*P* molecule this should correspond to approx $1/6\ 10^{-13}$ mole, in the case of a fully ionized molecule Since there will be some uncertainty as to the degree of ionization, the ion population at the microelectrode tip and the relative electrophoretic mobility of Glc-6-*P* ions as compared to other ions (H^+, Na^+, *etc*), for practical considerations the above figure is rounded off to $1\ 10^{-14}$ mole Glc-6-*P* for a 1-sec duration of a $1\ 10^{-8}$ A current This value when divided by a cell volume of about 5–10 pl (estimated for an average cell thickness of 5–10 μ) gives an initial intracellular concentration of 1–2 mM (which agrees with the physiological levels of glycolytic intermediates in ascites cells treated with saturating amounts of glucose)

A rough estimate of the average rate of substrate utilization is then possible in μmole/sec per kg wet wt from the estimated amount of added substrate and the fluorescence pulse halftime ($t_{1\ 2off}$) (NAD^+ reduction and reoxidation halftime) according to the formula[15]

$$k_3 E = \frac{S}{t_{1/2off}}$$

(k_3 = velocity constant of the decomposition of the enzyme–substrate complex, E = enzyme concentration, S = substrate concentration) Also the rate of substrate utilization can be alternatively expressed in A/pl per sec For a cell with a density around 1, the correspondence is roughly 1 μmole Glc-6-*P*/sec per kg wet wt = $1\ 10^{-12}$ amp /pl per sec or $1\ 10^{-12}$ coulomb/pl per sec[2]

RESULTS

A typical fluorescence curve recorded from the cytoplasm or nucleus of an EL2 cell (extramitochondrial space) with Glc-6-*P* as the substrate has been already described in detail[3–6] The same general pattern is observed in several hundreds of cells maintained under standard conditions Each intracytoplasmic addition of substrate is followed by a cycle of NAD^+ reduction and reoxidation (fluorescence pulse[4])

The modalities of substrate administration

When dose response curves are made (Figs 1 and 2A) starting from micro-

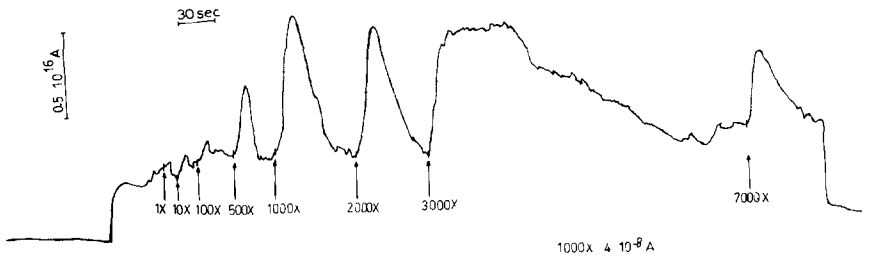

Fig 1 Microfluorimetric recording of the fluorescence changes in the extramitochondrial (nuclear) region of an EL2 cell in response to repeated microelectrophoretic additions of Glc-6-*P* — ATP in gradually increasing amounts (see arrows) 1 corresponds to a microelectrophoretic current of 4 10^{-11} A The time scale proceeds from left to right The baseline with both apertures of the differential microfluorimeter on free space is seen at both ends of the tracing The initial level of of fluorescence prior to the first addition of substrate is seen on the left of the first arrow (1) (Expt NPI/68) The magnitude of the primary photocurrent is indicated in 1 10^{-16} A

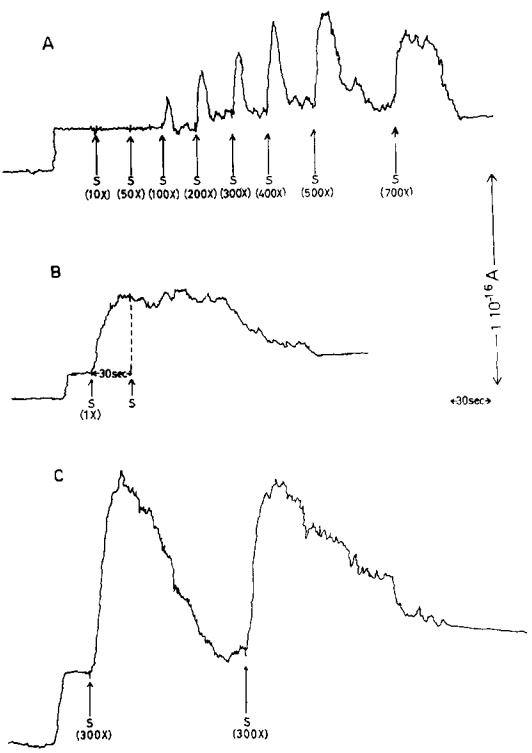

Fig 2 Microfluorimetric recordings illustrating the influence of the method used for the microelectrophoretic addition of glycolytic substrate Conditions are like in Fig 1 (except for ADP instead of ATP in the glycolytic combination) A Dose-response curve with stepwise increasing amounts of substrate (1-sec additions) B Continuous addition of minimal amounts The time of addition (30 sec) is indicated between the two S arrows C Optimal additions at once (1 sec) The current level for application in C was selected slightly below that required for a maximal response in A (Expt W5/68)

electrophoretic currents around 1 10^{-12} A at first barely detectable fluorescence pulses are recorded, which change little with substrate concentration However, beyond a certain point both the peak amplitude and the area of the pulses start increasing quite rapidly and exponentially Thus, for a doubling of substrate concentration in this region, there can be a 4–9 (or even 16) times increase in pulse area (Fig 3)

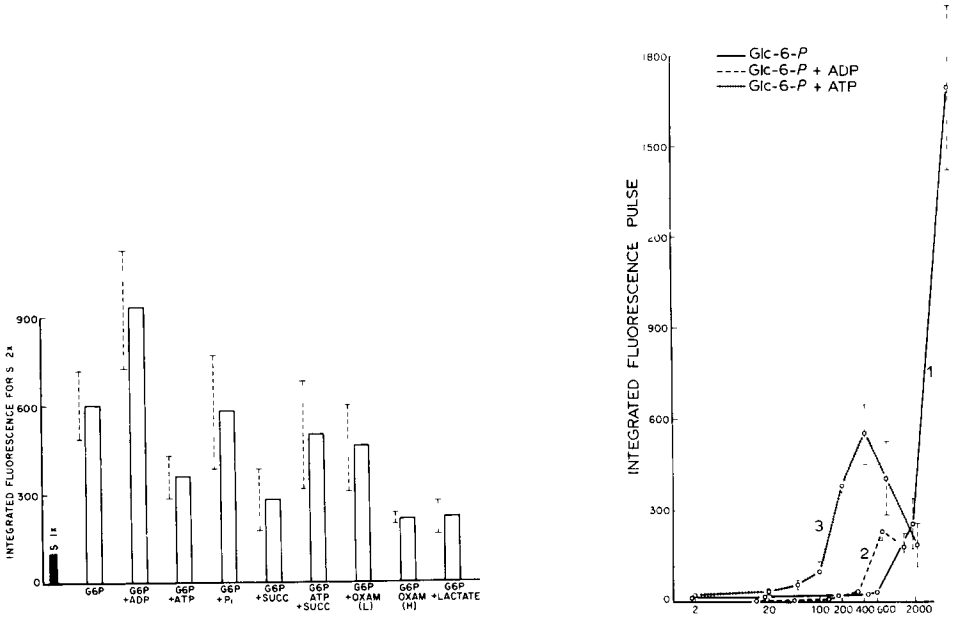

Fig 3 The rate of increase in NADH accumulation (expressed by the area of the fluorescence pulse) when the substrate (Glc-6-*P*) concentration *S* is doubled in the optimum region of the dose-response curve The height of the column in black corresponds to the pulse area for S = 1 × The height of the columns in white corresponds to the pulse area for S = 2 × with various glycolytic combinations as indicated SUCC = succinate, OXAM = oxamate S E for the pulse area calculated according to the formula $S.E. = \sqrt{\Sigma d^2/n(n-1)}$ The integrated area of the pulse corresponds to $\int_0^\infty P_1 dt$, P_1 being the pulse amplitude of the instant t

Fig 4 The relationship between the integrated fluorescence pulse (whole area of the pulse = $\int_0^\infty P_1 dt$) and the microelectrophoretic current (as a measure of the added Glc-6-*P*) in EL2 cells treated comparatively with three glycolytic combinations (Glc-6-*P* alone and with ATP or ADP) $\int_0^\infty P_1 dt$ is expressed in arbitrary units The microelectrophoretic current is expressed in 1 10^{-11} A/pl and plotted on a logarithmic scale S E was calculated like in Fig 3 (the curves are sigmoid on a linear scale also)

If substrate concentration is plotted against pulse area (Fig 4) the ascending branch of the resulting sigmoid curve[4] corresponds to the critical concentration range for which NAD^+ reduction rises the most rapidly After the peak amplitude of the fluorescence response is attained, with subsequent larger doses of substrate there is still a further increase in pulse area mainly due to considerable prolongation of the pulse halftime However, the rate of substrate utilization as calculated from the pulse

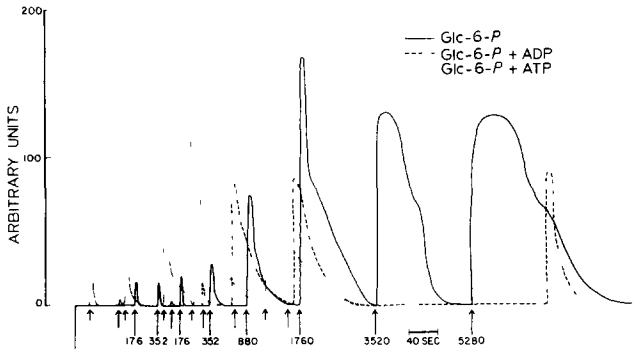

Fig 5 Summated dose-response curves for Glc-6-*P*, Glc-6-*P* + ADP and Glc-6-*P* + ATP in EL2 cells Each curve corresponds to the summation of determinations in at least 5 cells S E for the peak fluorescence levels in the most significant pulses is indicated in Table II The fluorescence levels are indicated in the ordinate in arbitrary units The numbers in the abscissa correspond to the added Glc-6-*P* in terms of 1 10^{-11} A/pl The time scale for the pulses is separately indicated and it proceeds from left to right The level of fluorescence prior to the first addition of substrate is seen on the left of the first arrow and it is arbitrarily equated to 0 Each arrow corresponds to a microelectrophoretic addition ↑ = Glc-6-*P*, ↑ = Glc-6-*P* + ADP, ↑ = Glc-6-*P* + ATP (Expts S1, 2 and 3/69)

halftime tends to level off or decrease Finally if substrate is increased further inhibition is observed with decrease of both peak amplitude and pulse area, until all responses cease

When maximal or submaximal levels of substrate are added at once (Fig 2C)

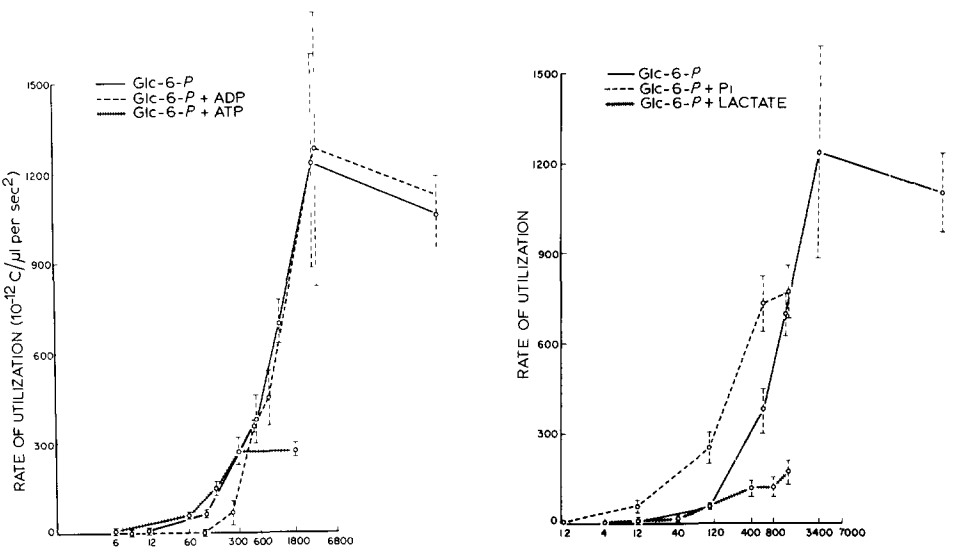

Fig 6 The rate of Glc-6-*P* utilization alone and in presence of adenine nucleotides, as calculated from the halftime of NAD^+ reduction and reoxidation after substrate addition (pulse halftime $t_{\frac{1}{2}\,off}$) The rate of substrate utilization is indicated on the ordinate in 1 10^{-12} coulomb/pl per sec^2 The values on the abscissa indicate the added substrate in 1 10^{-11} A/pl

Fig 7 Like in Fig 6 but for Glc-6-*P* alone *versus* in presence of P_i and lactate

TABLE I

RATES OF SUBSTRATE UTILIZATION

The rates are calculated from the halftime ($t_{\frac{1}{2}off}$) of the fluorescence pulse (see MATERIALS AND METHODS) and expressed in $1 \cdot 10^{-12}$ A/pl per sec for EL2 cells with an average thickness of 5 μl (the same values correspond also to the rate of substrate utilization in μmole/sec per kg wet wt for a rate of microelectrophoretic substrate ejection of $1 \cdot 10^{-14}$ mole per $1 \cdot 10^{-8}$ A, roughly). For dose curves optimal substrate doses are defined as the amounts required to elicit a fluorescence pulse of maximal amplitude (maximal NAD^+ reduction). S.E. is calculated according to the formula $S.E. = \sqrt{\Sigma d^2/n(n-1)}$

Conditions	*Optimal additions at once**	*Dose-response curves*	
		Optimal	*Supraoptimal*
Glc-6-*P*	1050 ± 260	1040 ± 370	990 ± 370
Glc-6-*P* + ADP	1020 ± 500	685 ± 200	605 ± 50
Glc-6-*P* + ATP	180 ± 50	150 ± 20	225 ± 45
Glc-6-*P* + P_i	1020 ± 200	420 ± 70	325 ± 60
Glc-6-*P* + succinate	440 ± 85	300 ± 70	415 ± 200
Glc-6-*P* + ATP + succinate	670 ± 280	140 ± 35	125 ± 25
Glc-6-*P*(5) + oxamate (1)	360 ± 220	100 ± 55	50 ± 15
Glc-6-*P*(1) + oxamate (1)	260 ± 50	40 ± 20	55 ± 30
Glc-6-*P* + lactate	240 ± 35	100 ± 10	120 ± 25

* Generally in continuous additions slightly lower rates were observed

a much higher NAD^+ reduction and considerably larger NADH accumulation can be attained, as compared to dose-response curves

When substrate is added continuously (Fig. 2B) the rise time of the NAD^+ reduction curve is 2–3 times longer (around 15 sec against 4–5 sec for the first two methods), there is a much longer duration of the steady state at peak reduction level (about 90 sec against less than 10) and as a result a considerable prolongation of the reoxidation halftime (120 sec)

TABLE II

PROPERTIES OF DOSE-RESPONSE CURVES

The average primary photocurrent recorded from the extramitochondrial region of an EL2 cell in a substrate and drug-free medium is about $1 \cdot 10^{-16}$ A. All values in the table are expressed accordingly

Conditions	*Minimal responses*			*Half maximal pulses*			*Maximal pulses*		
	Primary photocurrent (10^{-16} A)	*Substrate threshold (10^{-11} A/pl)*	*$t_{\frac{1}{2}off}$ (sec)*	*Primary photocurrent (10^{-16} A)*	*Substrate (10^{-11} A/pl)*	*$t_{\frac{1}{2}off}$ (sec)*	*Primary photocurrent (10^{-16} A)*	*Substrate (10^{-11} A/pl)*	*$t_{\frac{1}{2}off}$ (sec)*
Glc-6-*P*	1.2 ± 0.1	176	4	1.7 ± 0.1	880	10	2.7 ± 0.3	1760	16
Glc-6-*P* + ADP	1.2 ± 0.1	340	4				2.0 ± 0.1	680	17
Glc-6-*P* + ATP	1.2 ± 0.1	14	8	1.5 ± 0.1	56	10	2.2 ± 0.2	140	14
Glc-6-*P* + P_i	1.3 ± 0.1	8	5	1.8 ± 0.1	400	10	2.6 ± 0.3	800	18
Glc-6-*P* + oxamate	1.2 ± 0.1	15	7	1.4 ± 0.2	30	7	1.9 ± 0.2	75	13
Glc-6-*P* + lactate	2.0 ± 0.2	5	15	2.0 ± 0.2	5	15	3.0 ± 0.5	250	27

When the average rate of substrate utilization is calculated from the NAD^+ reduction–reoxidation halftime ($t_{1/2off}$) it appears that the rates (Table I) are often 2–4 times larger in maximal additions at once than the highest rates calculated from dose-response curves, while the rates for continuous delivery are in between

The influence of adenine nucleotides on metabolic transients

The concentration range at which fluorescence pulses start being recorded from the extramitochondrial space of EL2 cells, following the addition of Glc-6-*P* is lowered in presence of adenine nucleotides or P_i (Figs 5, 6 and 7, Table II) Specially for Glc-6-*P* + ATP substrate inhibition is reached at levels much below those required for a maximal response with Glc-6-*P* alone

The maximal levels of NAD^+ reduction are quite comparable in cells treated with Glc-6-*P* alone or supplemented with P_i (peak fluorescence responses at 167 and 191% over initial value, respectively) There is a certain decrease in the maximal amplitudes of the fluorescence pulses in presence of ATP and ADP (Fig 5, Table II) However, little correlation is found between the pulse amplitudes *per se* and the rates of substrate utilization, as calculated from the pulse halftimes for two fluorescence pulses of comparable amplitude and halftime, the higher rate will correspond to the pulse obtained with the larger substrate concentration (as a measure of substrate utilization per unit time) The rates of substrate utilization are about 4 times smaller in ATP-treated cells with regard to Glc-6-*P* alone, since the fluorescence maxima are reached for much lower concentrations in presence of ATP There is considerable parallelism (Figs 6 and 7) between the rates of substrate utilization in Glc-6-*P*, Glc-6-*P* + ADP and Glc-6-*P* + P_i-treated cells (around 700–1200 to ^{12}A/pl per sec)

The plots of substrate concentration against the integrated fluorescence pulse (whole area of the pulse $= \int_0^\infty p_1 dt$) show similar sigmoid curves[4] for Glc-6-*P* alone or supplemented with adenine nucleotides (Fig 4) as well as P_i However, the ascending branch of the sigmoid is displaced to the left in presence of adenine nucleotides of P_i, the maximum displacement occurring with ATP

Addition of succinate to Glc-6-*P* leads initially to little change in the kinetics of the fluorescence response However if EL2 cells are starved for over 60 min the response to Glc-6-*P* ceases almost totally in presence of succinate This response can be restored by supplementing the mixture with ATP (ATP/succinate ratio = 1/5)

The response to substrate and inhibitors of lactic dehydrogenase

In presence of oxamate[16–18] or lactate (Fig 7, Tables I and II) it is noteworthy that the main effect is on the rate of Glc-6-*P* utilization which drops considerably, while the level of Glc-6-*P* dependent NAD^+ reduction does not reflect consistently this change in rate (see preceding section) In all these cases, there is a considerable lowering of the Glc-6-*P* level required for maximal NAD^+ reduction (Table I) In presence of either lactate or oxamate, the reduction of glycolytic NAD^+ proceeds more effectively at suboptimal substrate concentrations, as compared to Glc-6-*P* alone However, at maximal substrate concentrations lactate differs from oxamate with lactate the NAD^+ reduction is equal to or over the highest values recorded for Glc-6-*P* alone, while with oxamate it is 30–50% lower Although theoretically oxa-

mate should have no effect on or enhance NAD^+ reduction (as it really does at suboptimal substrate levels), at maximal Glc-6-*P* (and oxamate) concentrations, the NAD^+ reduction may be adversely affected by the oxamate inhibition of glycolysis In these cells when metabolic inhibition sets in there is a lowering of the maximal NAD^+ reduction which can be obtained with glycolytic substrate (*cf* dose-response curve, Fig 2A) The sigmoid plots of the integrated fluorescence pulse against substrate concentration show a marked shift to the left for Glc-6-*P* supplemented with oxamate or lactate

DISCUSSION

The NADH/NAD^+ ratio and the equilibrium of the NAD^+-coupled enzymes

In these microfluorimetric experiments there are indications that the NAD^+-reducing system is somewhat more efficient than the NADH-reoxidizing when the cell is overloaded with glycolytic substrate (*e g* , at the end of a dose curve or with supraoptimal additions at once) the considerable prolongation of the NADH reoxidation time (or the persistance for several minutes of a plateau at peak reduction) demonstrate the inability of lactic dehydrogenase to cope with NADH as fast as it is being produced by glyceraldehyde phosphate dehydrogenase However, at optimal or suboptimal levels of substrate, lactic dehydrogenase is quite efficient in reoxidizing the accumulated NADH, as can be seen from the brief duration of the steady state at peak reduction (4–8 sec, Figs 1, 2 and 7) and the relatively short halftime for reoxidation

Also in the presence of oxamate and lactate which inhibit NADH reoxidation or favour the backward reaction at lactic dehydrogenase, the recording of NADH reduction by glyceraldehyde phosphate dehydrogenase is facilitated, with minimal or suboptimal doses of substrate (but the rate of Glc-6-*P* utilization is decreased[18] through interference with glycolytic flux[19])

The glycolytic flux

The glycolytic flux can be affected by the appearance of metabolic block[20–22] at various points along the glycolytic chain either through accumulation of allosteric inhibitors or exhaustion of activators

The larger peak reduction levels, NADH accumulation and higher rates of substrate utilization obtained with optimal additions at once or continuous flow of minimal doses (as compared to dose-response curves), correspond to situations in which either no time is allowed for metabolic block to set in (the former) or the substrate flow is kept below levels required for the block (the latter) In most cases the inhibition is preceded by a sometimes considerable prolongation of the halftime for reoxidation As to the inhibition itself, the accumulation of ATP[22–25], and diphosphoglycerate[22] (inhibitor of glyceraldehyde phosphate dehydrogenase) or the lack of both ADP and P_i (refs 20–22) have been incriminated in the past

Enzyme activity in vivo and in vitro

Computer simulation studies[23] of ascites cell metabolism have had to assume for reasons of simplicity that enzymes *in vivo* act as they do *in vitro* and that mass-action kinetics are valid for all these reactions In these microfluorimetric experiments,

the rates of substrate utilization calculated from the NAD^+ reduction–reoxidation halftime fall in the upper range (see Table I) of values obtained by other methods. Therefore there is no gross evidence to contradict the computer model. However, the kinetics of the fluorescence response to Glc-6-*P* in the extramitochondrial space of EL2 cells (Fig. 4) favour a higher-order relationship[7,8] with regard to substrate. Even enzymes which are seen to exhibit simple Michaelis kinetics *in vitro* may be acting as polymers *in vivo*[8] (in which case the exponent in the rate of increase in enzyme activity with substrate concentration, could provide a clue to the number of subunits[7,8] (see Fig. 3).

The effect of adenine nucleotides and P_i

The Glc-6-*P* threshold required for maximal NAD^+ reduction, the accumulation of NADH after addition of Glc-6-*P* and the rate of Glc-6-*P* utilization are all considerably lowered in presence of ATP. The ATP inhibition[22–24] of the glycolytic flux can be the result of action at several points along the glycolytic chain. Both ATP and ADP lead to a comparable decrease in the accumulation of NADH during metabolic transients which follow addition of Glc-6-*P*. However, the level of NAD^+ reduction is not a faithful indicator of the glycolytic rate in these cells. With ATP the rate is considerably smaller, but in presence of ADP or P_i the rate curves are strikingly parallel to the curve for Glc-6-*P* alone.

It is hard to pinpoint a single activator of glycolysis which might be of primordial importance in these EL2 cells. It may be that under the conditions of these experiments the EL2 cells are already quite self-sufficient in terms of activators of the glycolytic chain (*e.g.*, ADP and P_i), so that they depend only on the supply of substrate and will even stop utilizing it once they have replenished their ATP storage (thus explaining the ATP inhibition).

As far as the ATP dependence of the Glc-6-*P* response in presence of succinate and the lowering of the glycolytic rate with succinate, they might be due to competition for metabolites[26,27] (*e.g.*, adenine nucleotides) and interactions[26–29] between the mitochondrial and glycolytic compartments.

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