Biochimica et Biophysica Acta, 590 (1980) 107—116
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BBA 47803

RELATION BETWEEN ENERGY PRODUCTION AND ADENINE NUCLEOTIDE METABOLISM IN HUMAN BLOOD PLATELETS

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(Received June 19th, 1979)

Key words: Energy production; Adenine nucleotide metabolism; Adenylate energy charge; (Platelets)

Summary

The relation between ATP production and adenine nucleotide metabolism was investigated in human platelets which were starved by incubation in glucose-free, CN^--containing medium and subsequently incubated with different amounts of glucose. In the absence of mitochondrial energy production (blocked by CN^-) and glycogen catabolism (glycogen almost completely consumed during starvation), lactate production increased proportionally with increasing amounts of glucose. The generated ATP was almost completely consumed in the various ATP-consuming processes in the cell except for a fixed portion (about 7%) that was reserved for restoration of the adenylate energy charge. During the first 10 min after glucose addition, the adenine nucleotide pool remained constant. Thereafter, when the glycolytic flux, measured as lactate formation, was more than 3.5 $\mu mol \cdot min^{-1} \cdot 10^{-11}$ cells, the pool increased slightly by resynthesis from hypoxanthine-inosine and then stabilized; at a lower flux the pool decreased and metabolic ATP and energy charge declined to values found during starvation. Between moments of rising and falling adenylate energy charges, periods of about 10 min remained in which the charge was constant and ATP supply and demand had reached equilibrium. This enabled comparison between the adenylate energy charge and ATP regeneration velocity. A linear relation was obtained for charge values between 0.4 and 0.85 and ATP regeneration rates between 0.6 and 3.5 ATP equiv. $\cdot min^{-1} \cdot 10^{-11}$ cells. These data indicate that in starved platelets ATP regeneration velocity and energy charge are independent and that each appears to be subject to the availability of extracellular substrate.

Introduction

The concept of the adenylate energy charge (= (ATP + 1/2 ADP)/(ATP + ADP + AMP), Ref. 1) as the key regulator of cellular energy metabolism has received almost general acceptance. Experimental evidence in favor of such a role is based on the following observations: (1) many types of cells varying from *Escherichia coli* [2,3] to mammalian blood platelets [4] exhibit adenylate energy charges of about 0.9, as predicted by the model; (2) starvation of *E. coli* by glucose exhaustion lowers the energy charge and reduces various functional capacities, e.g. protein synthesis [3]; (3) many regulatory enzymes in vitro are sensitive to alterations in the energy charge and respond in accordance with the concept that a high-energy charge facilitates energy-consuming sequences but retards energy-producing pathways and vica versa [5]. Conclusive evidence, however, can only be obtained from rate measurements of energy-producing and consuming sequences in intact cells in which the energy charge is varied and this is technically difficult. The opposite approach, that is varying the rates of energy production and consumption while measuring the energy charge is easier, but requires cell suspensions in which ATP regeneration and consumption can be varied quantitatively.

Suspensions of gel-filtered human platelets, in which mitochondrial ATP production is blocked by CN^-, respond to changes in the extracellular glucose concentration with predictable variations in glycolytic and glycogenolytic flux [6]. The absence of creatinephosphate [7] makes the generated ATP the only carrier for transfer of energy from sites of generation to sites of utilization. This so-called metabolic ATP (in contrast to granular, non-metabolic ATP, Ref. 8) can be accurately measured with isotopic tracer techniques since platelets lack significant de novo synthesis of adenine nucleotides [8]. From the sum of ATP regeneration from ADP and accumulation, the rate of ATP consumption can then be deduced.

In these suspensions we studied the relation between the adenylate energy charge and ATP regeneration during a period in which the platelets were starved by exhaustion of glucose and glycogen and were subsequently incubated with different amounts of glucose thereby restoring glycolytic ATP regeneration. It will be shown that during the restoration phase, the cell reserves a fixed part of the generated energy for restoration of the adenylate energy charge. Once equilibrium is reached between ATP supply and demand, the charge stabilizes at levels directly proportional to the rate of ATP regeneration, indicating that during starvation substrate availability becomes the main factor regulating ATP resynthesis, thereby excluding control by the energy charge.

Materials and Methods

Freshly drawn venous blood (40-ml portions) was collected from healthy volunteers into citrate (0.1 vol. of 129 mM citrate). After centrifugation ($200 \times g$, 10 min, 22° C) the supernatant, platelet-rich plasma, was incubated with 1 μM [U-^{14}C]adenine (spec. act. 286 Ci · mol^{-1}, Radiochemical Center, Amersham) for 30 min at 37° C to label the metabolic pool of the adenine

nucleotides [9]. The platelets were then isolated by gel filtration at 22° C [10] on Sepharose-2B (Pharmacia, Uppsala, column size 5 cm × 20 cm) equilibrated in Ca^{2+}-free Tyrode's solution (pH 7.2, osmolarity 300 mosM, Ref. 11) containing 1 mM KCN and no glucose. Under these conditions mitochondrial energy production and glucose catabolism are absent, leaving glycogen as the only source for ATP regeneration [6]. After gel filtration was completed (within 20—30 min) platelets were incubated at 37° C in the same medium.

pH corrections were made every 5 min (with 0.1 N HCl) to prevent changes beyond the range pH 7.2—7.5 due to CO_2 liberation. At different times, samples were collected for measurement of lactate and ethanol-soluble [^{14}C]-adenine nucleotides in total cell suspensions, as described earlier [6]. Tests for cell lysis [6] were negative throughout the incubation period. Total radioactivity in [^{14}C]adenine nucleotides, IMP and inosine plus hypoxanthine remained constant. For further details see Ref. 6.

Results

Platelets were gel-filtered in glucose-free, CN^--containing Tyrode's solution and subsequently incubated in the same medium. During the first 40—50 min incubation, glycogen was slowly catabolized to lactate. Concomitantly, [^{14}C]-ATP fell and [^{14}C]AMP increased thereby rapidly lowering the adenylate energy charge [6]. During subsequent incubation lactate production ceased and [^{14}C]ATP and [^{14}C]AMP stabilized, indicating that a period of no detectable ATP generation and consumption began which the cells sustained for another 60 min before any cell lysis could be detected. Between 0 and 75 min incubation, lactate formation could be restored to values of non-starved platelets (3.60 ± 1.26 μmol · min^{-1} · 10^{-11} cells, mean ± S.D., $n = 12$, Ref. 12) by addition of 1000 μM glucose or more (Fig. 1). After longer incubation, glucose-

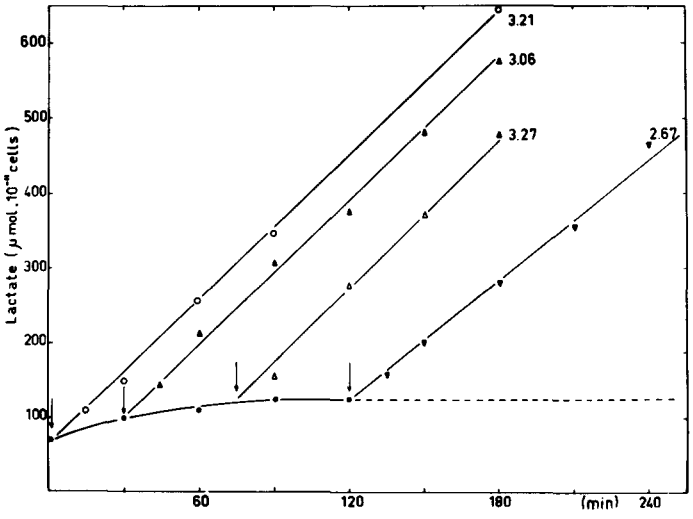

Fig. 1. Lactate formation (μmol · 10^{-11} cells) in gel-filtered platelets ($1.6 \cdot 10^8$ cells · ml^{-1}) incubated in glucose-free medium containing 1 mM CN^-. Arrows indicate the addition of 1000 μM glucose. Numbers give glycolytic flux (μmol lactate formed · min^{-1} · 10^{-11} cells).

induced lactate formation was slower, indicating that irreversible alterations in glucose-converting sequences had taken place. The effect of glycolytic energy production on adenine nucleotides and adenylate energy charge was therefore investigated after incubating the cells for 75 min in substrate-depleted medium.

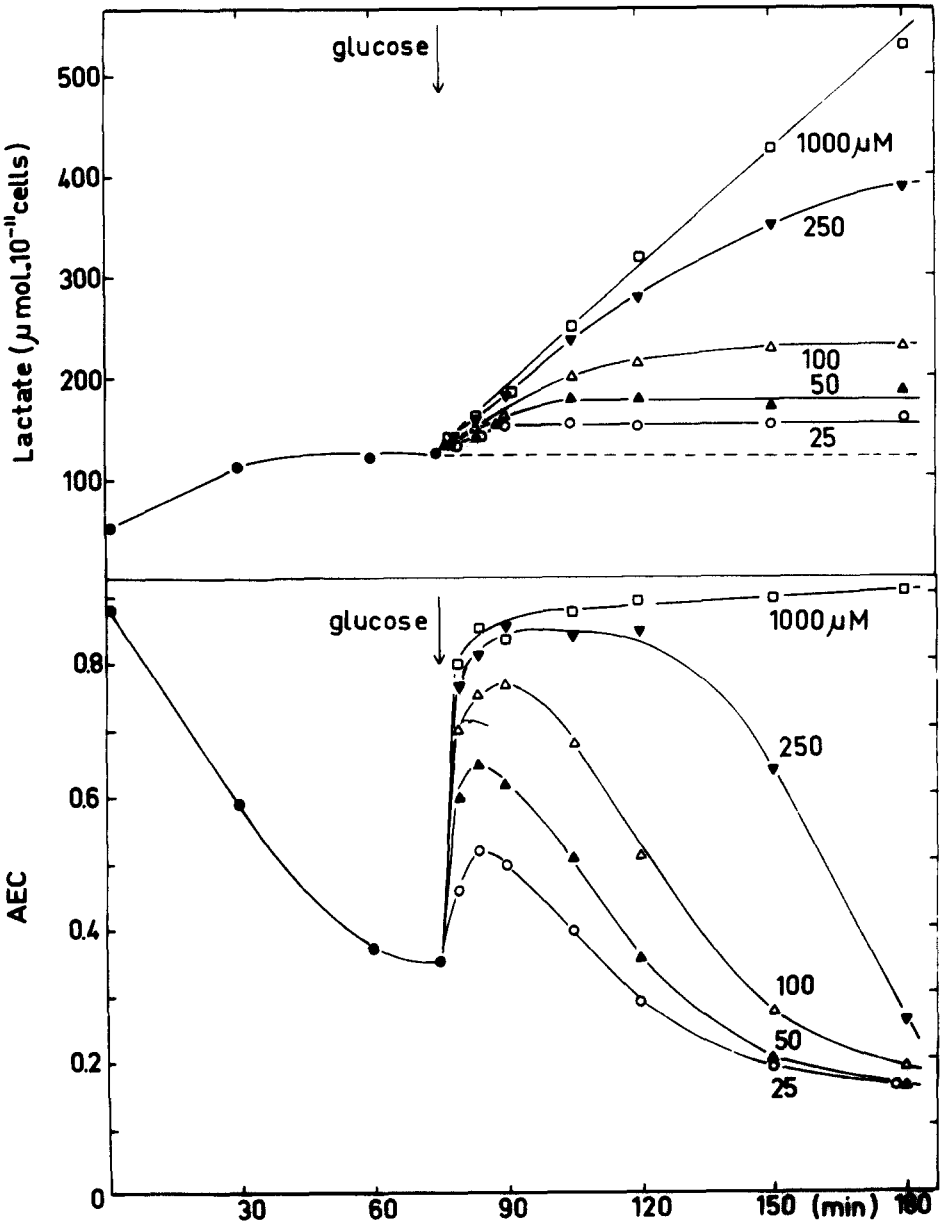

Fig. 2. Lactate formation ($\mu mol \cdot 10^{-11}$ cells), [^{14}C]adenine nucleotides and metabolites (percent of total ^{14}C radioactivity) and adenylate energy charge (AEC) in gel-filtered platelets (2.10^8 cells · ml^{-1}) incubated in glucose-free CN^--containing medium. The arrows indicate the additions of 25 µM (○———○), 50 µM (▲———▲), 100 µM (△———△), 250 µM (▼———▼) and 1000 µM (□———□) glucose. [^{14}C]IMP data were not included and varied between 7 and 11% of total radioactivity.

As more glucose was added, lactate production was faster and reached a maximum of 3.12 μmol lactate formed $\cdot$ min^{-1} $\cdot$ 10^{-11} cells at 1000 μM glucose (Fig. 2). Thereafter, lactate production declined as more and more glucose was metabolized. The concomitant variations in [^{14}C]adenine nucleotides showed a

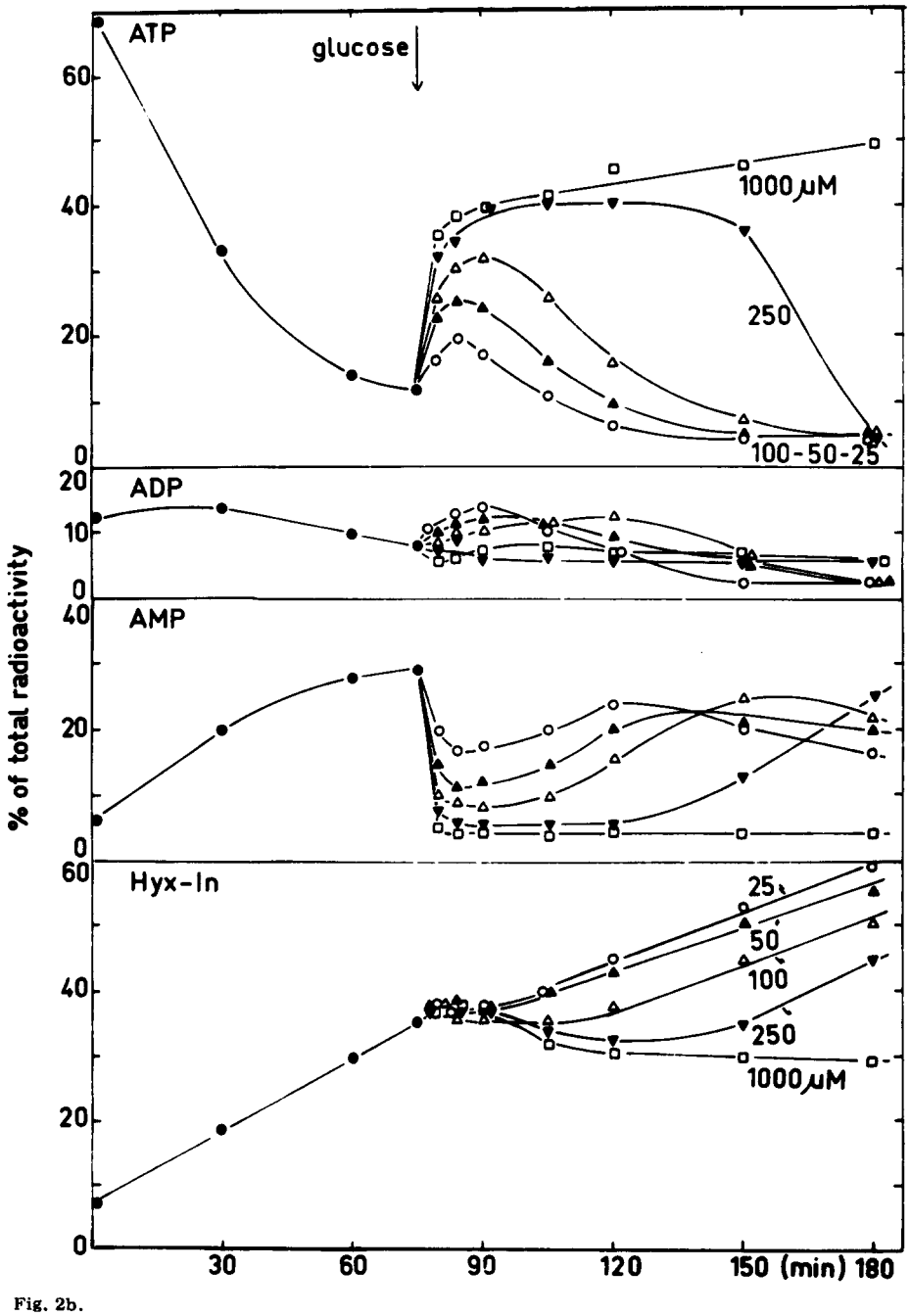

Fig. 2b.

biphasic pattern. During the first 15 min the total ^{14}C radioactivity in ATP + ADP + AMP remained constant (47.2 ± 1.2, mean ± S.D., n =16 in Fig. 2, and 47.9 ± 2.6 and 49.1 ± 1.2% of total ^{14}C radioactivity in two other separate experiments). Thereafter, ^{14}C-labelled (ATP + ADP + AMP) gradually increased when lactate formation was rapid (greater than 3.5 μmol lactate formed · $min^{-1} \cdot 10^{-11}$ cells) but sharply declined when lactate formation was slow or absent. Upon glucose addition, both adenylate energy charge and [^{14}C]ATP levels rapidly increased and reached higher values when more glucose induced a higher rate of lactate formation. When the glycolytic flux declined, both energy charge and [^{14}C]ATP levels fell again. [^{14}C]ADP and [^{14}C]IMP (not shown) varied within small limits. Variations in [^{14}C]AMP were a reflection of the changes in [^{14}C]ATP, but remained below about 30% of total radioactivity. [^{14}C]Inosine-hypoxanthine was constant during the first 15 min after glucose addition. Thereafter, this fraction increased when lactate formation was slow or absent. When lactate production was more than 3.5 μmol · $min^{-1} \cdot 10^{-11}$ cells, however, [^{14}C]inosine-hypoxanthine levels slightly decreased and then stabilized (Fig. 2).

From the variations in the levels of the [^{14}C]adenine nucleotides and concomitant lactate production during the first 10 min after glucose addition, the relation between production of energy and energy accumulation in adenine nucleotides was calculated. During this period [^{14}C]ATP levels and adenylate energy charge rose at all glucose concentrations. Since the sum of ^{14}C-labelled (ATP + ADP + AMP) did not change in this period, variations in [^{14}C]ATP, [^{14}C]ADP and [^{14}C]AMP were the sole result of the adenylate kinase reaction and glycolytic ATP generation. The latter was calculated from the rate of lactate formation. Because no change in glycogen level could be detected and aerobic metabolism was inhibited by CN^-, 1 mol of lactate formed equalled the conversion of 1 mol of ADP into 1 mol of ATP, that is the formation of one ATP equivalent (ATP equiv.; Ref. 5). The [^{14}C]ATP radioactivity was converted to μmol · 10^{-11} cells by assuming that [^{14}C]adenine incubation of non-starved cells labels the metabolic adenine nucleotides homogeneously and that variations in ^{14}C radioactivity therefore reflect changes in the concentrations of metabolic adenine nucleotides [12,13]. Variations in [^{14}C]-adenine nucleotides could thus be converted into absolute terms and expressed as ATP equiv. (Table I). As more glucose induced a faster glycolytic flux, more [^{14}C]AMP was converted into [^{14}C]ATP. [^{14}C]ADP slightly accumulated at low rates, but fell back to initial levels at higher rates of lactate formation. This means that more ATP equiv. accumulated in metabolic ATP as the glycolytic flux rose. Compared to the rate of ATP equiv. formation, the amount of ATP equiv. that accumulated remained constant, suggesting that a fixed part of the generated energy accumulated (about 7%, Table I, and 7.3 ± 0.7% and 7.3 ± 0.4%, mean ± S.D., in two other suspensions). This caused a rapid recovery of the adenylate energy charge at still very low [^{14}C]ATP values. At 1000 μM glucose the charge rose from 0.35 to 0.85, close to the preincubation value (0.87). At the same glucose concentration [^{14}C]ATP levels rose from 12 to 40% of total radioactivity, only 58% of the preincubation value

At a lower glycolytic flux, restoration of the energy charge was less complete and as soon as the flux declined, the charge decreased again. Between

TABLE I

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Data derived from Fig. 2 for the first 10 min after glucose addition. Variations is [^{14}C]adenine nucleotides were converted into absolute amounts on the basis that the 80% of total ^{14}C radioactivity found in ATP in normal platelets corresponds to the total metabolic ATP concentration (3.5 μmol · 10^{-11} cells; Refs. 12 and 13). Energy data were expressed in ATP equiv. [5] reflecting an energy accumulation of 1 equiv. for the ADP → ATP and of 2 equiv. for the AMP → ATP conversion. Energy production was calculated from lactate production which was solely glycolytic (1 μmol lactate ≈ 1 μmol ATP equiv.). Data between brackets are mean ± S.D.

Glucose (μM)	% of total radioactivity variations in ^{14}C-labelled:			Energy turnover (ATP equiv./10 min)			
				Accumulation	Production	Consumption	Accumulation/ production (%)
	ATP	ADP	AMP	(μ mol · 10^{-11} cells)			
25	+8	+4	−12	0.88	12.0	11.0	7.3
50	+14	+3	−18	1.36	20.8	19.5	6.5
100	+19	+2	−21	1.75	23.2	21.5	7.5
250	+24	0	−24	2.10	31.2	29.1	6.7
1000	+26	−1	−25	2.23	31.2	29.0	7.2
							(7.0 ± 0.4)

stages of rising and falling energy charges, periods of 10—15 min (depending on the glycolytic flux) remained in which [^{14}C]ATP and [^{14}C]AMP were constant and ATP supply and demand apparently had reached equilibrium (Fig. 2). More precise sampling in these periods enabled the measurement of

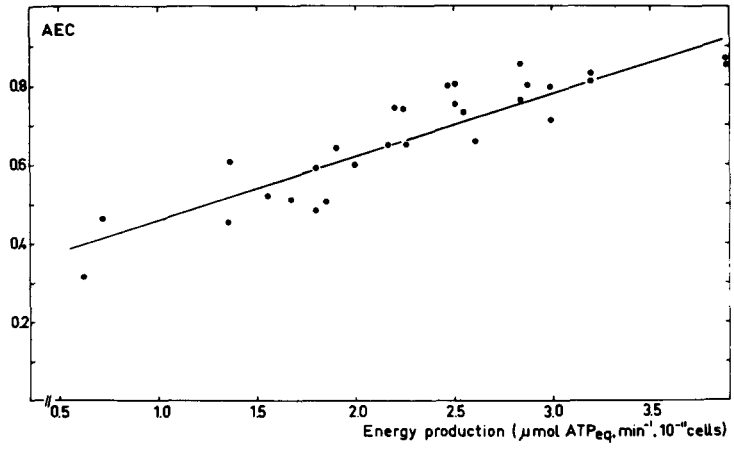

Fig. 3. Relationship between adenylate energy charge (AEC) and energy production (μmol ATP equiv. · min^{-1} · 10^{-11} cells) in gel-filtered platelets recovering from a period of starvation (incubation in glucose-free, CN^--containing medium) after addition of various amounts of glucose. Data were calculated from experiments similar to the one described in Fig. 2, but with sampling (n = 5) for lactate and [^{14}C]adenine nucleotides at 3-min intervals in the regions where energy charge and rate of lactate formation were constant. Data (n = 29) were derived from seven suspensions. Each separate suspension showed the same linearity between energy charge and energy production rate. Regression line with correlation coefficient: $y = 0.16x + 0.30$, $r = 0.90$.

[^{14}C]adenine nucleotides and concomitant lactate formation over a broad range of ATP-regeneration velocities. From these data the relationship between adenylate energy charge and ATP regeneration velocity could be calculated. A linear correlation was obtained between energy charges varying from 0.4 to 0.85 and ATP resynthesis rates varying from 0.6 to 3.5 μmol ATP equiv. · $min^{-1} \cdot 10^{-11}$ cells (Fig. 3).

Discussion

The present data show that human platelets can be starved by incubation for 75 min in a glucose-free, CN^--containing medium without loosing their cell integrity. Those cells even sustain a period with no lactate production or changes in [^{14}C]ATP, ADP and AMP. When starvation is terminated by glucose addition, they resume the formation of lactate (Fig. 1), normalize their adenylate energy charge (Fig. 2) and perform normal dense body secretion and aggregation in the presence of the proper stimulus (Akkerman, J.W.N. et al., unpublished results). A period of complete standstill of detectable ATP generation and consumption apparently does not induce immediate cell death. However, minor amounts of ATP might well be regenerated in other processes than glycolysis thereby keeping the cells viable. The correlation between ATP generation and adenine nucleotide metabolism made after a period of starvation is therefore probably close to the situation in normal platelets in which variations in energy charge and ATP supply are less excessive.

Upon glucose addition, lactate formation is restored and the generated ATP is almost entirely directed to the various ATP-consuming processes in the cell. A fixed part of the generated ATP (about 7%) is, however, reserved for converting [^{14}C]AMP into [^{14}C]ATP, thereby rapidly restoring the adenylate energy charge. This part is independent of the absolute rate of ATP resynthesis and also at low rates of ATP production part of the ATP is withheld for the AMP → ATP conversion. Apparently, restoration of the energy charge has a high priority even in conditions where the various ATP-consuming sequences function far below optimal capacity.

During the first 10 min after glucose addition, the total adenine nucleotide pool remains constant and the restoration of the adenylate energy charge is thus the sole result of the adenylate kinase reaction and glycolytic ADP phosphorylation. During this period the adenylate kinase reaction shifts in the direction of ADP formation with a velocity proportional to the rate of lactate formation, that is to the ADP → ATP conversion. This velocity seems therefore to be regulated by the disappearance of ADP or the formation of ATP. Since [^{14}C]ADP levels hardly changed, the ATP level seems rate limiting for the adenylate kinase reaction in the direction of ADP formation.

When during subsequent incubation the glycolytic flux remains high (greater than 3.5 μmol lactate formed · $min^{-1} \cdot 10^{-11}$ cells) thereby keeping the energy charge above 0.8 and [^{14}C]ATP above 40% of total radioactivity, [^{14}C]inosine-hypoxanthine is incorporated into the adenine nucleotide pool. In platelet suspensions hypoxanthine is almost entirely extracellular [14] and can be taken up and further metabolized via the salvage pathway [15]. Using the uptake kinetics of normal platelets [15] and similar calculations as those used

for [^{14}C]ATP (Table I), reuptake of hypoxanthine in our studies would label less than 2% of the metabolic pool in 1 h. The decrease in [^{14}C]hypoxanthine-inosine, for about 80% consisting of hypoxanthine [14], suggests that hypoxanthine incorporation can be about ten times faster in conditions of a low adenine nucleotide pool and a normal glycolytic flux.

At a lower glycolytic flux restoration of the energy charge is slower and as soon as the flux declines, energy charge, [^{14}C]ATP and total adenine nucleotide pool start to decline at about the same rate as in the first 75 min starvation period. Since [^{14}C]IMP levels remained constant and [^{14}C]hypoxanthine reuptake is small, the increase in [^{14}C]hypoxanthine-inosine reflects loss of adenine nucleotides by deamination of AMP. In vitro, platelet AMP deaminase is activated by lowering the adenylate energy charge although a certain concentration of ATP is required for optimal activity [16]. In the intact platelet, AMP was deaminated during the first 75 min at approximately constant velocity, despite the changes in the levels of the substrate [^{14}C]AMP and the activator [^{14}C]ATP. During the first 15 min after glucose addition no AMP deamination could be detected, probably as the result of rapid AMP phosphorylation in the adenylate kinase reaction. Thereafter, AMP deamination resumed when the energy charge dropped below 0.65 in accordance with the in vitro properties of the enzyme. Despite the variations in energy charge and [^{14}C]ATP, ADP and AMP during this period, deamination occurred at almost the same velocity, indicating that regulation of AMP deaminase is poor.

Between moments of increasing and decreasing energy charges periods of about 10 min remained in which [^{14}C]ATP and [^{14}C]AMP were constant and ATP generation and utilization had reached equilibrium. This enabled us to compare the adenylate energy charge with various rates of ATP production. A linear relationship was obtained between charges varying form 0.4 to 0.85 and ATP resynthesis rates varying from 0.6 to 3.5 μmol ATP equiv. formed $\cdot$ $min^{-1} \cdot 10^{-11}$ cells. The energy charge is therefore a valuable indicator of the rates of ATP production and consumption and can reach normal values at low absolute levels of adenine nucleotides, which confirms earlier observations [4]. Surprisingly, the energy charge equally responded to the rate of ATP resynthesis over the whole range from 0.3 to 0.85. Regulatory enzymes that participate in sequences in which ATP is resynthesized ('R systems', Ref. 5) usually respond to a fall in energy charge by an increase in catalytic activity whereas control enzymes in sequences in which ATP is used ('U systems') are inhibited. Those enzymes show the highest responsiveness to charges varying between 0.8 and 0.9 but are relatively insensitive to changes in the energy charge in the lower range. ATP-producing and consuming sequences thereby stabilize the energy charge at about 0.9 and such a value has indeed been found in many types of cells [2—4,17—19]. The correlation between energy charge and 'R' and 'U' systems has never been demonstrated in the intact cell, mainly caused by lack of means to change the energy charge without affecting the cell's energy metabolism. The opposite approach used in our studies does not reveal special areas where the rate of ATP resynthesis is more responsive to the energy charge than in others; in fact, over the whole range between 0.4 and 0.85 ATP resynthesis and energy charge respond in parallel to the changes in extracellular glucose concentration. Apparently within this range ATP

regeneration velocity and energy charge are independent and each appears to be subject to the availability of extracellular glucose.

The data therefore indicate that in starved platelets substrate availability becomes the dominant factor controlling ATP regeneration velocity, thereby excluding control by the energy charge over a wide range (about 0.4—0.85) of charge values. Below and above this range data are difficult to obtain and control by the charge within the narrow range between 0.85 and 0.95 might well occur, as predicted on theoretical grounds [5]. Furthermore, the figures show that within the range of 0.4—0.85 stable energy charges can be found when ATP production and consumption are in equilibrium. ATP homeostasis apparently does not automatically involve a high (greater than 0.90) energy charge, as was predicted previously [5].

Acknowledgement

The authors thank Dr. H. Holmsen, Specialized Center for Thrombosis Research, Temple University, Philadelphia, U.S.A., for careful criticism of the manuscript.

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