BBA 45909

CONTROL OF ENERGY METABOLISM IN PLATELETS. A COMPARISON OF AEROBIC AND ANAEROBIC METABOLISM IN WASHED RAT PLATELETS

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(Received November 3rd, 1969)

SUMMARY

1. To obtain information about control of energy metabolism in platelets, washed rat platelets were incubated under oxygen or nitrogen, either without glucose or with 4 mM glucose.

2. Respiration inhibited glycolysis by 75% without glucose and by 50% with glucose. Glucose inhibited respiration by 50% and stimulated glycolysis from 2- to 4-fold.

3. Insulin had no effect on glucose metabolism.

4. In the absence of glucose, glycogen consumed was accounted for as lactate produced under aerobic as well as anaerobic conditions. With glucose, all glucose *plus* glycogen consumed was not accounted for as lactate, but the stoichiometry was the same with both aerobic and anaerobic incubations. These data are interpreted as indicating oxidation of some endogenous substrate other than glucose or glycogen.

5. Without glucose, the ratio ATP/ADP declined during anaerobic incubation. The ratio ATP/ADP was higher with glucose than without.

6. Under aerobic conditions, oxidative phosphorylation accounted for about 55% of total ATP production with glucose and almost 90% without glucose.

7. Comparison of mass action ratios with apparent equilibrium constants indicated phosphofructokinase (EC 2.7.1.11) and pyruvate kinase (EC 2.7.1.40) as potential control enzymes. Changes in levels of metabolites with altered flux indicated that phosphofructokinase was a major glycolytic control under the conditions of these experiments.

8. Citric acid cycle intermediates were measured, but no control points could be identified.

INTRODUCTION

Blood platelets have an energy requirement for routine maintenance *plus* an additional requirement for their special functions in maintaining hemostasis. This additional requirement is observed as an increased rate of metabolism after addition

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of thrombin, or other agents that cause platelet aggregation and viscous metamorphosis, to a suspension of platelets. There has been disagreement concerning the relative contributions of aerobic and anaerobic metabolism to the overall energy metabolism of platelets[1–3]. There have also been conflicting reports about the types of changes associated with the increased metabolism following addition of thrombin[2,4–7].

Energy metabolism and its control in other tissues have been successfully investigated by comparing levels of metabolic intermediates under different steady-state conditions. Rate limiting reactions, and therefore potential control points, are identified as those for which the "mass action ratio" (calculated as an equilibrium constant using levels of reactants found at steady-state conditions) is much less than the apparent equilibrium constant. Evidence that these steps are actually involved in control is obtained by measurement of metabolites under different metabolic fluxes (the overall rate of the pathway). A control point is indicated by a lower substrate level at the higher flux.

A previous study[9] of the levels of glycolytic and citric acid cycle intermediates in washed rat platelets indicated mass action ratios near equilibrium constants except for phosphofructokinase (EC 2.7.1.11) and pyruvate kinase (EC 2.7.1.40). This paper reports the effects of changes in metabolic flux on levels of glycolytic and citric acid cycle intermediates in platelets. Different metabolic fluxes were established by incubating platelets under either nitrogen or oxygen with or without glucose. Metabolic flux was determined by measuring the rates of change of glycogen, glucose, pyruvate, lactate, and oxygen. Evidence is presented that a higher glycolytic flux in anaerobic platelets is associated with activation of phosphofructokinase and possibly pyruvate kinase.

METHODS

Preparation of platelet suspensions

Rat blood was obtained and platelets were isolated as previously described[9] except that the blood was diluted with 0.5 vol. of 0.9% NaCl to improve the yield of platelets. Platelets were washed twice in about 10 ml of cold incubation mixture and were finally suspended in a volume of incubation mixture sufficient to give a suspension of about $5 \cdot 10^9$ platelets per ml. The incubation mixture was Krebs–Ringer phosphate solution[10] without $CaCl_2$ but containing 1 mM EDTA, either with 4 mM glucose or without glucose. Without added glucose, the level of glucose in the suspensions was less than 1% of the level of glycogen and was therefore ignored.

Incubation of platelet suspensions

Platelets were incubated at 37° in the chambers of an oxygen electrode instrument*. The suspensions were stirred with a magnetic stirrer. The chambers were covered with rubber stoppers through which a tube carried a steady stream of gas, nitrogen for anaerobic experiments and oxygen for aerobic experiments. A 1-mm slot in the stopper permitted gas to escape without permitting mixing of the chamber contents with the atmosphere.

* Model 53, Biological Oxygen Monitor, Yellow Springs Instrument Co., Yellow Springs, Ohio.

After placing the platelet suspension in the incubation chamber, 3 min was allowed for temperature and gas equilibration before taking initial samples for extraction and measurement of glycogen, glucose, lactate, pyruvate, ATP, ADP and AMP. Final samples were taken for extraction and measurement of the above compounds and intermediates of glycolysis and the citric acid cycle after an additional incubation for 10 min without glucose or 15 min with glucose. The longer incubation with glucose was necessary to obtain measurable changes in the large amount of added glucose. During aerobic incubation, a Clark-type oxygen electrode was inserted into the suspension for 2-min periods for recording of oxygen consumption.

Extraction and measurement of metabolites

The methods of extraction and analysis by enzyme fluorometric assays were the same as used previously[9]. Glucose and lactate were measured by enzyme spectrophotometric procedures[11]. To avoid metabolic changes during sampling, samples were removed with rapid automatic pipets*, so that only a few seconds was required to withdraw the sample and mix it with the extracting solution.

A previous study[9] demonstrated a large variability in levels of metabolites among different platelet preparations, so that comparisons are best made with two treatments of one preparation. However, it was difficult to obtain enough platelets in one preparation to do the entire series of analyses for both aerobic and anaerobic platelets. Therefore only a few experiments were carried out this way and the data were averaged with the others. But it was clear from these few experiments that the major differences found by comparing two treatments on different preparations would have been found with less variability if all experiments had been done with two treatments on one preparation.

RESULTS

Glycolytic flux was estimated from depletion of glycogen *plus* glucose and production of lactate *plus* pyruvate between the initial and final extractions. The results are shown in Table I as rates, the changes assumed to be linear during the 10- or 15-min incubation period. Aerobic oxidation was estimated from the rate of oxygen consumption, also shown in Table I. The following conclusions can be made from the data in Table I. (i) Respiration inhibits glycolysis, by 75 % without glucose and by 50 % with 4 mM glucose. This is the Pasteur effect, and has been previously reported[12] in platelets. (ii) Glucose inhibits respiration by about 50 %, with a stimulation of glycolysis of over 2-fold under anaerobic conditions and about 4-fold under aerobic conditions. This is the Crabtree effect and has also been reported with platelets[6]. (iii) Insulin has no effect on glycolysis or respiration in washed rat platelets. This contrasts with the report of KARPATKIN[12] that insulin caused a 3-fold increase in glycolysis in washed human platelets. We therefore repeated these experiments with human platelets and again found no effect of insulin. (iv) Without added glucose, glycogen consumed can be accounted for by production of lactate, two lactate produced for each glucosyl unit lost. This is expected for anaerobic metabolism, but not for aerobic metabolism, where pyruvate would be expected to be oxidized through

* Eppendorf Push–Button Microliter pipet, Brinkman Instruments, Inc.

Biochim. Biophys. Acta, 197 (1970) 117–126

TABLE I

CHANGES IN LEVELS OF GLYCOLYTIC SUBSTRATES AND PRODUCTS AND OXYGEN DURING INCUBATION OF WASHED PLATELETS UNDER VARIOUS CONDITIONS

The experimental procedures are described under METHODS. Values are nmoles/10^{10} platelets per min ± S.E. Where indicated, 0.4 unit insulin per ml was added.

Conditions of incubation	*Number of Expts.*	*Substrates*			*Products*			*Oxygen*
		Glycogen	*Glucose*	*Total*	*Lactate*	*Pyruvate*	*Total*	
No glucose								
Anaerobic	7	−114 ± 8	—	−114 ± 8	227 ± 13	1 ± 0.5	228 ± 13	
Aerobic	6	− 29 ± 4	—	− 29 ± 4	72 ± 14	7 ± 0.9	79 ± 16	−108 ± 3
4 mM glucose								
Anaerobic	4	− 37 ± 9	−251 ± 14	−288 ± 18	516 ± 41	2 ± 0.8	518 ± 42	
Aerobic	4	− 15 ± 5	−132 ± 10	−147 ± 17	254 ± 23	6 ± 0.8	260 ± 23	− 58 ± 8
4 mM glucose plus insulin								
Anaerobic	5	− 20 ± 7	−224 ± 10	−244 ± 14	444 ± 18	2 ± 0.9	446 ± 18	
Aerobic	5	0	−120 ± 16	−120 ± 16	178 ± 4	7 ± 0.5	185 ± 5	− 52 ± 8

the citric acid cycle instead of being reduced to lactate. To confirm this observation, separate experiments were carried out using less concentrated platelet suspensions and longer incubation periods, with samples taken for analysis at 3, 13, and 23 min. From 75 to 105% of glycogen consumed could be accounted for as lactate, and changes where essentially linear for the entire period. These data apparently indicate that some endogenous substrate other than glycogen is the substrate for the oxygen consumed.

With glucose added to the incubation mixture, the stoichiometry is not as clear, possibly because of the difficulty of accurately measuring small changes in the large amount of added glucose. Although lactate production does not fully account for consumption of glucose *plus* glycogen, the stoichiometry is the same in aerobic and anaerobic incubations, evidence that the oxygen consumption was at the expense of substrates other than glycolytic end products.

TABLE II

ENERGY STATUS OF PLATELETS INCUBATED UNDER VARIOUS CONDITIONS

The data are from the experiments of Table I. The time of incubation before extracts were made is shown. The values are means ± S.E.

Condition of incubation	*Number of Expts.*	*Ratio ATP/ADP*	
		3 min	*13 min*
No glucose			
Anaerobic	8	3.1 ± 0.3	2.2 ± 0.1
Aerobic	7	3.3 ± 0.3	3.2 ± 0.1
		3 min	*18 min*
4 mM glucose			
Anaerobic	5	3.6 ± 0.5	3.4 ± 0.4
Aerobic	5	3.8 ± 0.4	3.9 ± 0.5

The energy status of the platelets at the time extracts were made is indicated by the ratios ATP/ADP in Table II. Without glucose, anaerobic platelets were unable to maintain a constant ratio ATP/ADP as evidenced by a decline ($P < 0.02$) during the brief incubation period. There was no decline with aerobic incubation. With 4 mM glucose in the incubation medium, the ratios were slightly higher than without glucose and they did not change during the incubations. When all the ratios with glucose are compared with the ratios without glucose (but omitting the anaerobic values at 13 min), the difference is statistically significant ($P < 0.05$), suggesting that washed platelets maintain a different "energy status" depending on substrate availability. HOLMSEN[13] has demonstrated compartmentation of adenine nucleotides in platelets, with a metabolic pool that exchanges with [^{32}P]orthophosphate and a non-metabolic, or storage pool, that does not. If the ratio ATP/ADP of only the metabolic pool changes with changing metabolism[14], the changes would actually be much greater than shown in Table II for total adenine nucleotides.

The relative contributions of glycolysis and oxidative phosphorylation to plate-

TABLE III

THEORETICAL ATP PRODUCTION BY GLYCOLYSIS AND OXIDATIVE PHOSPHORYLATION IN PLATELETS UNDER VARIOUS CONDITIONS

Rates of ATP production were calculated from data in Table I. Calculations were based on 2 ATP for each glucose and 3 ATP for each glucosyl unit of glycogen converted to lactate and 3 ATP for each 0.5 O_2 consumed. Units are nmoles ATP/10^{10} platelets per min.

Conditions of incubations	*Theoretical ATP production*			
	Glycolysis		*Oxidative phospho-rylation*	*Total*
	Glycogen	*Glucose*		
No glucose				
Anaerobic	343	—	—	343
Aerobic	87	—	648	735
4 mM glucose				
Anaerobic	111	502	—	613
Aerobic	45	264	348	657
4 mM glucose plus insulin				
Anaerobic	60	448	—	508
Aerobic	0	240	312	552

let energy metabolism under the different conditions of incubation are estimated in Table III*. Oxidative phosphorylation accounts for about 55 % of total ATP production with glucose and almost 90 % without glucose. Under anaerobic conditions, the same total ATP production can be maintained by glycolysis alone when glucose is available but not when glycogen is the only available substrate (*cf.* Table II). The required glycogen utilization would lead to depletion of glycogen stores within 5 min.

Comparison of apparent equilibrium constants with mass action ratios under the incubation conditions of these experiments (Table IV) confirms the previous observations[9] with cold platelets that phosphofructokinase and pyruvate kinase catalyze non-equilibrium reactions and are therefore potential control points. The lower mass action ratio for phosphoglucomutase when glucose is not available suggests that it may be a partially limiting step when glycogen is the only substrate for glycolysis. This could account in part for the greater glycolytic capacity with glucose.

The effects of altered glycolytic flux on steady-state levels of glycolytic intermediates are shown in Fig. 1, showing anaerobic levels as percent of aerobic levels. Thus Fig. 1A shows the effect of the 4-fold increase in glycolytic flux associated with

* ROCK AND NEMERSON[20] have suggested that oxidative phosphorylation is uncoupled in washed platelets, so that calculations of theoretical yields of ATP production would not be valid. They based their conclusion on the observation that labelled succinate, citrate, and palmitate were oxidized to CO_2 without preventing a decline of ATP and without increasing the rate of incorporation of labelled inorganic into organic phosphate. But the rate of phosphate incorporation with oxidation of exogenous substrates would be increased only if the oxidation of these substrates caused an increase in total oxidation rate which was not measured. The observation of the Crabtree and Pasteur effects and the fact that the ATP to ADP ratio was maintained with a lower glycolytic flux in anaerobic platelets demonstrate that the washed platelets in these experiments had coupled oxidative phosphorylation.

TABLE IV

COMPARISON OF APPARENT EQUILIBRIUM CONSTANTS OF SOME ENZYMES OF GLYCOLYSIS AND THE CITRIC ACID CYCLE WITH THEIR MASS ACTION RATIOS UNDER VARIOUS CONDITIONS

Mass action ratios were calculated as product over substrate with the reaction in the direction glycogen to CO_2. The data are the same as for Figs. 1 and 3.

Enzyme		*Apparent equilibrium constant**	*Mass action ratios*			
			No glucose		*4 mM glucose*	
			Anaerobic	*Aerobic*	*Anaerobic*	*Aerobic*
Phosphoglucomutase	(EC 2.7.5.1)	18	2.4	6.2	15	17
Phosphoglucoisomerase	(EC 5.3.1.9)	0.28	0.31	0.22	0.25	0.29
Phosphofructokinase	(EC 2.7.1.11)	$1.2 \cdot 10^3$	1.4	0.5	0.9	0.3
Phosphoglyceromutase	(EC 2.7.5.3)	0.1–0.17	—	0.36	—	—
Phosphopyruvate hydratase	(EC 4.2.1.11)	4.6–6.3	—	1.7	—	—
Pyruvate kinase	(EC 2.7.1.40)	$2 \cdot 10^3$–$15 \cdot 10^3$	<25**	<60**	<18**	<72**
Aconitate hydratase	(EC 4.2.1.3)	0.07	0.019	0.013	0.028	0.018
Fumarate hydratase	(EC 4.2.1.2)	4.0	4.1	3.9	7.6	5.6

* Values used by others[19,21] for similar comparisons.
** Calculated on the basis of total pyruvate in the suspension, including extracellular pyruvate.

anaerobiosis in the absence of glucose and Fig. 1B shows the effect of a 2-fold increase due to anaerobiosis with 4 mM glucose.

There is no generally accepted method of interpretation of data of this type. Some workers (*e.g.* see refs. 15 and 16) consider the "crossover" theorum[17] to be of at least partial applicability, so that control by phosphofructokinase would be indicated in Figs. 1 A and B, since the substrate (fructose-6-*P*) is decreased and the product (fructose-1,6-P_2) is increased. Other workers consider the crossover theorum invalid for systems such as glycolysis, since it was developed for systems, such as the electron transport chain, where only the ratio of "substrate" to "product" can change and not the total amounts (see review by SCRUTTON AND UTTER[18]). NEWSHOLME AND GEVERS[8] consider only the substrate of a non-equilibrium reaction to be important, a control site indicated by a change in substrate opposite the change in flux. This interpretation of Fig. 1A would suggest control by both phosphofructokinase and pyruvate kinase, since both their substrates (fructose-6-*P* and phosphoenolpyruvate) are decreased with increased flux. Fig. 1B would indicate control only by phosphofructokinase, but this type of data can never prove that an enzyme is not involved in control. The higher level of glucose-1-*P* in anaerobic platelets is consistent with activation of phosphorylase and the more rapid breakdown of glycogen indicated in Table I.

Half the experiments of Fig. 1B were carried out with 0.4 unit insulin per ml. The data were not apparently different from that obtained without insulin, further evidence for the lack of effect of insulin.

Fig. 2 shows the effect of glucose on the levels of glycolytic intermediates. Addition of 4 mM glucose increased glycolytic flux by 2.5 times in anaerobic platelets and 5 times in aerobic platelets. In each case, the change is associated with increased intermediates from glucose-6-*P* to dihydroxyacetone-*P*. Since the changes are in the same direction as the flux, they do not indicate control points. The direct correlation

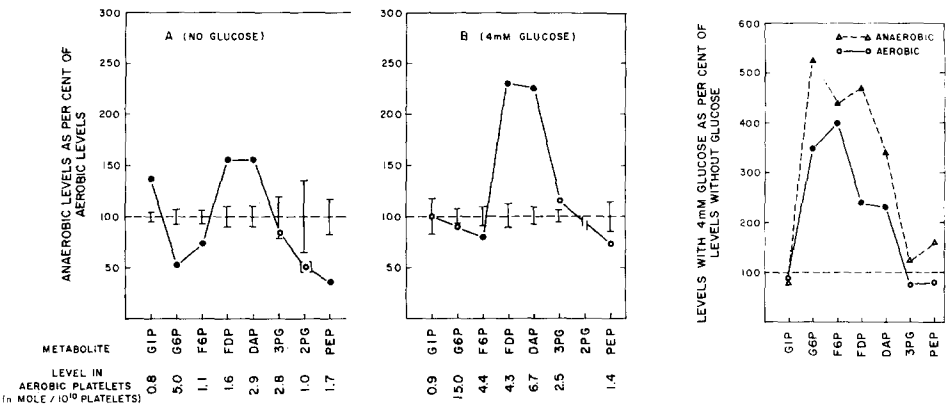

Fig. 1. Levels of glycolytic intermediates in anaerobic platelets as percent of levels in aerobic platelets. Each point is the average of 4–8 experiments. The standard error of the mean for the aerobic levels is indicated by brackets on the 100% line. Solid symbols represent values that are significantly different ($P < 0.05$) from aerobic values. Abbreviations are: G1P, glucose-1-*P*; G6P, glucose-6-*P*; F6P, fructose-6-*P*; FDP, fructose-1,6-P_2; DAP, dihydroxyacetone-*P*; 3PG, 3-*P*-glycerate; 2PG, 2-*P*-glycerate; PEP, *P*-enolpyruvate. In incubations without glucose, anaerobic levels of 2-*P*-glycerate were too low to measure; the point shown in brackets is the minimum level that would be measured and thus represents the maximum level in anaerobic platelets. Reliable measurements of 2-*P*-glycerate were not obtained with platelets incubated with 4 mM glucose. A, without glucose; B, 4 mM glucose.

Fig. 2. Levels of glycolytic intermediates in platelets incubated with 4 mM glucose as percent of levels in platelets incubated without glucose. The data, abbreviations, and symbols are the same as for Fig. 1.

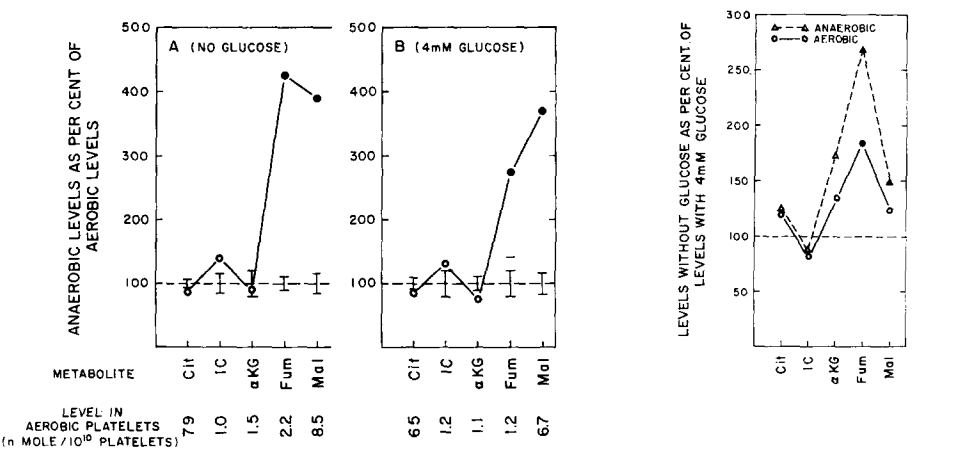

Fig. 3. Levels of citric acid cycle intermediates in anaerobic platelets as percent of levels in aerobic platelets. The experimental conditions and the treatment of data are the same as for Fig. 1. Abbreviations are: Cit, citrate; IC, isocitrate; αKG, α-ketoglutarate; Fum, fumarate; Mal, malate.

Fig. 4. Levels of citric acid cycle intermediates in platelets incubated without glucose as percent of levels in platelets incubated with 4 mM glucose. The data, abbreviations, and conventions are the same as for Fig. 3.

between flux and levels of fructose-1,6-P_2 and dihydroxyacetone-P in Figs. 1 and 2 may be due to a rate limiting reaction between dihydroxyacetone-P and 3-P-glycerate.

The effects of complete inhibition of the citric acid cycle by anaerobiosis on levels of intermediates are shown in Fig. 3. The only significant changes are the large increases in fumarate and malate. In contrast GOLDBERG *et al.*[19] measured the levels of citric acid cycle intermediates in mouse brain following decapitation which leads to anaerobiosis and found an increase in fumarate, but not malate, and a large decrease in α-ketoglutarate. In addition to the tissue difference, they observed the aerobic to anaerobic transition, their longest period being 30 sec after decapitation, whereas in these experiments a steady state has presumably been reached.

Location of control points requires the comparison of two fluxes, neither of which is zero. This should be possible by comparison of levels of citric acid cycle intermediates from aerobic incubations with and without glucose. This is shown in Fig. 4. Fumarate and malate are increased in the glucose-free medium, but this change also occurs in the anaerobic experiments so that it must be a direct result of glucose and not the consequence of a change of flux. Therefore these experiments give little information regarding control of citric acid cycle activity. A greater variation of flux is probably necessary.

DISCUSSION

The relative contribution of aerobic metabolism to the total energy metabolism of platelets has been questioned for many years. Although it is generally accepted that platelets have the capacity for aerobic metabolism, they are usually considered to be primarily a glycolytic tissue[1,3,6,12]. The results presented here indicate a large contribution of aerobic metabolism to total energy metabolism in platelets and possibly explain why previous studies have not led to this conclusion. The fact that a major part of glucose metabolized was converted to lactate led to the conclusion that platelets derive most of their energy from glycolysis. But the results of the present study indicate that even under conditions where all of the glycogen consumed was converted to lactate, aerobic metabolism was responsible for most of the ATP production, apparently by utilization of other endogenous substrates.

The observation of 100% conversion of glycogen to lactate in aerobic as well as anaerobic platelets incubated without glucose was at first surprising, but it does have support from published observations. For example, comparison of Figs. 4 and 5 of ref. 2, indicate that the rate of glucose oxidation to CO_2 is only about 1% the rate of lactate production. And although KARPATKIN AND LANGER[7] found only 55% conversion of either glucose or glycogen to lactate, they found that this relationship was not changed when glycolysis was stimulated by respiratory inhibitors. Thus their results would also suggest that respiration had been at the expense of substrates other than glucose.

While it is apparent that metabolites other than glucose or glycogen can be substrates for respiration, it is not clear what these substrates are. In the blood, platelets would have an essentially constant supply of several possible substrates, but with washed platelets suspended in a medium free of substrates, lipids seem like the most likely endogenous substrate reservoir. There is an obvious need to determine what endogenous substrates are available for platelet energy metabolism.

It was previously observed with washed rat platelets maintained at 0° (ref. 9), and confirmed here with platelets in more nearly normal metabolic conditions, that phosphofructokinase and pyruvate kinase catalyze non-equilibrium reactions and are therefore possible sites of control of glycolysis. The data in Fig. 1 clearly indicate that phosphofructokinase is a major control point. Fig. 1A suggests that pyruvate kinase is also a control enzyme, but until confirmed with more evidence, this should be a tentative conclusion. The data do not eliminate the possibility of other control steps, such as glyceraldehyde-3-*P*-dehydrogenase (EC 1.2.1.12) or 3-*P*-glycerate kinase (EC 2.7.2.3), where substrates were not measured, hexokinase (EC 2.7.1.1), where the intracellular level of glucose was not determined, and phosphorylase (EC 2.4.1.1).

These experiments have been analyzed with the assumption that glycolysis is the predominant pathway of glucose metabolism. The extent of the hexose monophosphate pathway in platelets has not been clearly established, but since nearly 100% of glycogen or glucose consumed was converted to lactate, it would appear to be slight in these experiments. The platelets capacity for gluconeogenesis is also not known, but under the conditions of these experiments it must be unimportant for interpretation of the data.

ACKNOWLEDGEMENTS

This research was supported by Grant HE 10099 from the National Heart Institute, U.S. Public Health Service, and was carried out with the technical assistance of Mr. Michael DeJesus, Jr.

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