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Steady-State Characteristics of Reconstituted Glycolytic Pathway and Regulation of Flux of Pathway by Cooperation of Metabolites and Enzymes.

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INTRODUCTION

Problems concerning with glycolysis and its regulation have been extensively studied, especially since the time when H.A. krebs advocated "pacemaker concept" for the control of metabolic pathways in 1957[1].

The rate of glucose utilization by cells is influenced by many factors. For instance, the effect of hormones such as insulin, glucagon, cortisol, and some drugs such as 2, 4-dinitrophenol, monoiodoacetate on glycolysis have been well documented for long time. On the other hand, the information on the pacemakers of glucose utilization, particularly through glycolysis, have drawn researcher's attentions to the role of adenylate compounds such as ATP, ADP and AMP as the regulators on the glycolysis.

The studies on enzymes and metabolites under steady state and transient conditions in living system or model system have also drawn attention to the key steps or key enzymes which are concerned with the regulation of metabolic pathways.

A vast amount of literatures have been reported on the metabolic control of glycolysis during 1960's. Most of them have been mainly related to oscillating mechanism of the concentration of intermediates[2-7], while some of them were concerned with partially reconstructed system of glycolysis[8-11]. They ascribed the main loci of control of glycolysis and generation of oscillation to the

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The following abbreviations have been used in this paper. ALD --- aldolase (EC 4.1.2.13), ADP --- adenosine diphosphate, AMP --- adenosine monophosphate, ATP --- adenosine triphosphate, ENO --- enolase (EC 4.2.1.11), GAPDH --- glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12), GDH --- glycerol-3-phosphate dehydrogenase (EC 1.1.1.8), GF --- glycolytic flux, HK --- hexokinase (EC 2.7.1.1), LDH --- lactate dehydrogenase (EC 1.1.1.27), MK --- myokinase (EC 2.7.4.3), PGI --- phosphoglucose isomerase (EC 5.3.1.9), PFK --- phosphofluctokinase (EC 2.7.1.11), PGK --- phosphoglycerate kinase (EC 2.7.5.3), PGM --- phosphoglycerate phosphomutase (EC 2.7.5.3), PK --- pyruvate kinase (EC 2.7.1.40), TIM --- triosephosphate isomerase (EC 5.3.1.1), NAD^+ --- nicotine adenine dinucleotide, NADH --- reduced nicotine adenine dinucleotide,

concerted feedback regulation at the steps of PFK and of GAPDH by adenine and pyridine nucleotides. However, many problems are still remained to be solved on the functionally active amounts of constituent enzymes of the pathway as the factors of controlling the rate of glycolysis under steady state and oscillatory state which are deeply correlated with the amounts of coenzymes, coenzyme potentials, cofactors, redox-state and so on.

This paper reports an analysis of the steady state characteristics in reconstructed glycolytic pathway using highly purified enzymes and coenzymes in terms of their control strength and sensitivity coefficients which characterize dynamical system.

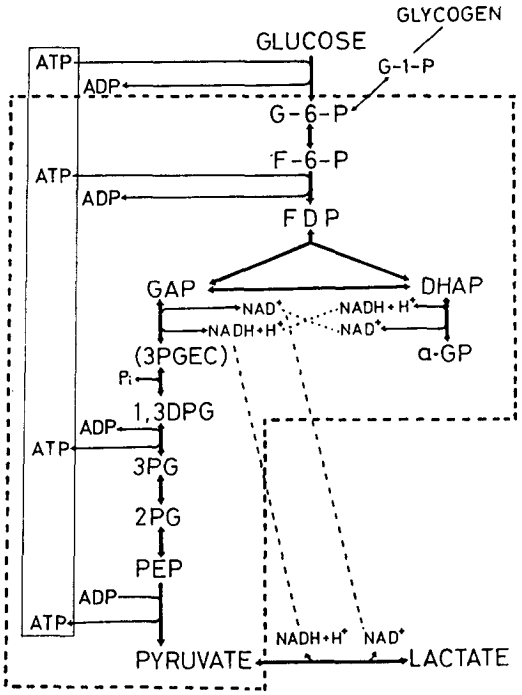

Fig. 1 Diagram of glycolytic system.

Pathway of glycolysis was reconstructed based on this diagram. The reactions were carried out first by the system encircled by broken line, i.e. so-called "Embden-Meyerhof pathway" proper for the case that the effects of adenine and pyridine nucleotide, NADH/NAD ratio, Pi, Mg^{++}, and K^{+} on GF were observed and secondly by the system to which HK as initiating enzyme and glucose as a substrate were added when the effects of ATP/ADP and enzyme profiles were estimated.

To initiate the reactions under the constant levels of NADH/NAD ratio, i.e. so-called "pyridine nucleotide potential" and ATP/ADP ratio, i.e. so-called "adenine nucleotide potential", it is necessary to reconstruct the systems without TIM which catalyzes the interconversion between GAP and DHAP in the presence of excess amount of α-glycerophosphate dehydrogenase. In other words, we are able to establish steady state equilibrium on ATP⇌ADP and NADH⇌NAD coupling which are repidly equilibrated by separating the glycolytic pathway at the initial step of triose-phosphate metabolism as it is shown in this diagram.

EXPERIMENTAL

1. Materials and Methods

Basic schema of the pathway

Fig. 1 shows the diagram of glycolytic system which is reconstructed as a multienzyme system by using purified enzymes and coenzymes. The system encircled by the broken line is a pathway predominantly present in cytosol. On the other hand, from the point of view of compartmentation glucose and lactate are separated from glycolytic pathway by the broken line because thay are present fairly large amount in extracellular fluid and often considered as different compartments, that is, the pools to supply substrates to the systems of carbohydrate metabolism by transfer reaction which is carried out by HK and LDH[12]. However, HK are also added to the system in the special case of estimating the effect of ATP/ADP, i.e., coenzyme potential on GF (refer to the explanatory note on Fig. 1).

Standared system of reconstructed glycolytic pathway

The reconstructed system is summarized in Table 1. The enzymes, adenine and pyridine nucleotides, and glycolytic intermediates were obtained from Boeringer Mannheim Yamanouchi KK. The sources and purity of the enzymes are shown in Table 2. The purity of enzymes was pure enough to reconstitute the glycolytic system because contaminations amongst the constituent enzymes were less than 0.1% each other.

To select and set the concentration of nucleotides and inorganic ions in the reconstructed systems we referred to physiological concentrations found in normal mammalian tissues and Michaelis constants of the enzymes[13].

GF which was also estimated and confirmed by pyruvate formation was measured by the absorbance changes of NADH at 340 nm, that is, the rate of NAD^+ reduction, spectrophotometrically as a routine in this experiment on a Hitachi Recording Spectrophotometer Type 124 and LKB reaction rate analyzer 8600.

The activity of each enzyme was also assayed by the rate of NAD^+ reduction and NADH dehydrogenation in advance to experimental use by coupled enzymatic analysis[14]. The initial concentrations of G-6-P and glucose in the reconstructed system were 10mM and 20mM, respectively in order to maintain the substrate at high level to keep steady state during observations (Table 2).

The experiments were carried out at 25°C.

Table 1.

Tris·HCl buffer	pH 7.4, 0.2M final volume 2.0 ml.
Enzymes	1 unit
Coenzymes	ATP: 1mM, 4mM ADP: 2mM, NAD: 2mM
Substrate	G-6-P: 10 mM or Glucose: 20mM
Phosphate	6.0 mM, 10mM
$MgSO_4$	2.4 mM
KCl	6.0mM
2,3-Diphospho-glycerate	0.05mM

Table 2.

Enzymes	Source	Purity
HK	Yeast	G-6-P-DH, GR, myokinase 6-PGDH each<0.01; PGI<0.1%
PGI	Yeast	F-6-PK, GR, 6 PGDH and PGLuM<0.01% each
PFK	Rabbit muscle	ALD (F-1, 6-P-spec) and ALD (F-1-P-spec), PGI<0.01% each; HK 0.03%
ALD	Rabbit muscle	GAP-DH 0.01% TIM<0.03%; GDH<0.05%
TIM	Rabbit muscle	ALD, GDH each 0.01%, GAP-DH<0.001%
GAPDH	Rabbit muscle	GDH<0.01% PFK PGM and TIM<0.05% each
PGK	Yeast	"NADH oxidase" 0.01%; GAP-DH GDH myokinase<each 0.01%; TIM<0.1%
PGM	Rabbit muscle	Enolase, PGK each<0.01%
ENO	Rabbit muscle	PGM, PK each<0.02%
PK	Rabbit muscle	Enolase, LDH, myokinase each<0.01%
LDH	Rabbit muscle	PK, aldolase each<0.001% GPT MDH myokinase each<0.01%
GDH	Rabbit muscle	ALD, GAPDH, LDH and TI Meach<0.01%
MK	Rabbit muscle	ATPase<0.01% PGK<0.02%

Table 1. Standard system of reconstructed glycolytic pathway
The concentrations of coenzymes and inorganic ions were decided according to physiological concentrations in tissues or cells. On one hand we also refered to Michaelis constant and data reported in the references cited in this paper. The amount of enzymes was decided considering their stability and activities enough for the observation.

Table 2. Enzymes used in the reconstruction of glycolytic pathway
The sources and purity are shown in the first and second column, respectively, which are described in the publication, "Biochemica information", Boehringer Mannheim GmbH 1973. Their specific activities were checked before use according to the methods descrived in "Methods of Enzymatic Analysis" ed. H.U. Bergmeyer, Verlag Chemie, Weinheim, 1973.

Estimations of flux control parameters.

Control strength and sensitivity coefficients of the constituent enzymes and cofactors were also determined. The definition of control strength and sensitivity coefficients in which the dynamical characteristics of the constituents of the pathway are reflected are as follows[15)16)]:

$$F_G = f(E_i, S_j, I_k, K_l) = f(a_1, a_2 \cdots\cdots a_N)$$

in a Steady state

where

F_G : Net flux of glycolysis/unit volume

a_i : Structural Control Variables(SCV)

$$a_i \begin{cases} E_i : \text{Enzymes} \\ S_j : \text{Substrates and Coenzymes} \\ I_k : \text{Inorganic compounds} \\ K_l : \text{Kinetic parameters.} \end{cases}$$

and

$$\text{Control Strength} : \frac{\partial F_G}{\partial a_i}$$

$$\text{Sensitivity Coefficient} : \frac{a_i}{F_G} \frac{\partial F_G}{\partial a_i}$$

RESULTS

Effect of inorganic phosphate and adenine nucleotides on the GF

Table 3 shows the effects of phosphate and AMP on the GF against the control experiments in which both of the compounds were not added at all. The flux increased in proportion to phosphate concentration, while AMP affected the flux strongly at the concentration of 0.1 mM and further addition did not show any influence on the flux. This would indicate the allosteric characteristic of AMP which is an allosteric activater to PFK[17)18)].

When other adenine nucleotides, i.e., ADP and ATP were added to the system, the flux increased in accordance with the nucleotide concentration as it is shown in Table 4. However, the GF rather decreased in the presence of 3.0 mM or more of ADP and ATP. This would be the results of competitive effects between ATP and ADP which are inhibitor and enhancer to PFK, respectively[18)].

Correlation between GF and ATP/ADP ratio (adenine nucleotide potential).

In order to confirm the effect of adenine nucleotides on the flux, we examined to reconstruct glycolytic pathway maintaining the ATP/ADP at constant ratios which is especially important in correlation with so-called phosphate potential ([ATP]/[ADP][Pi]), (refer to the explanatory note in Fig. 1).

		Glycolytic Flux (G.F.)	
		G.F. (μM/min)	%(Stimulation)
Control	Pi 0mM, AMP 0 〃, ADP 0 〃, ATP 4.0 〃	1.0	0
Pi (mM)	0.1	2.4	140
	0.2	3.4	220
	0.6	5.6	460
	2.0	6.9	590
Control	AMP 0mM, Pi 0 〃, ADP 0 〃, ATP 4.0 〃	1.0	0
AMP (mM)	0.1	5.6	460
	0.3	4.8	380
	0.5	5.0	400

Table 3. Effect of inorganic phosphate and AMP on GF
Experimental conditions were the same as standared system except for Pi and AMP.

		Glycolytic Flux (G.F)	
		G.F (μM/min)	% Stimulation
Control (AMP 0 mM, ADP 0, ATP 4.0, P_i 0.6)		5.8	
ADP (mM)	0.05	5.8	0
	0.1	5.8	0
	0.2	6.5	12.1
	0.5	7.3	25.9
	1.0	7.7	32.8
	2.0	10.6	82.8
	3.0	6.5	12.1
	4.0	8.1	39.7
ATP (mM)	0.05	8.2	-
	0.1	9.7	-
	0.5	16.1	-
	1.0	15.5	-
	1.5	19.4	-
	2.0	21.0	-
	3.0	17.3	-
	4.0	15.3	-
ATP/ADP (ATP+ADP=3.0 mM)	0.25 / 2.75	3.7	74
	0.5 / 2.5	6.2	116
	1.0 / 2.0	9.2	179
	1.5 / 1.5	11.8	493
	2.5 / 0.5	9.7	210

Pyruvate Formation from glucose (μM)

Table 4. Effect of ADP, ATP and adenine nucleotide potential on GF
Experimental conditions were the same as standared system except for the variables in this experiment.

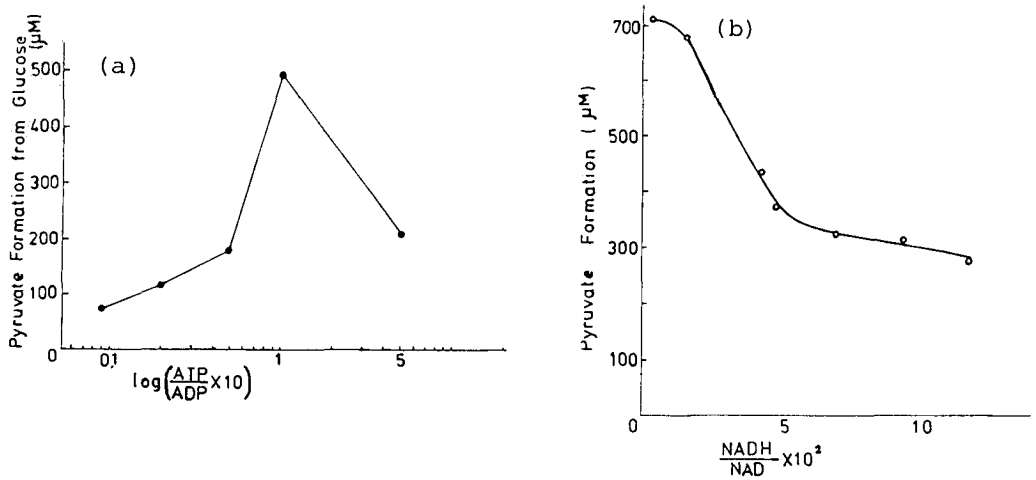

Fig. 2-a Effect of adenine nucleotide potential on reconstructed glycolytic system.
The plot represents pyruvate formation from glucose against the logarithm of [ATP/ADP] x 10.

Fig. 2-b Effect of pyridine nucleotide potential on the recohstructed system.
The plot was drawn based on the data in Table 5.
Experimental conditions were shown in Table 4 and Table 5, respectively.

The GF and the formation of pyruvate at the various levels of adenine nucleotide potentials are shown in Table 4. It is evident from the results that the flux and pyruvate formation from glucose increased with the increment of ATP/ADP ratio, showing a peak in the case of 1/1 ratio. This would be the special feature which is considered as the results of complete recycling from ATP to ADP and vice versa in glycolytic system, i.e., ATP⇄ADP is equilibrated, thus the GF turns into a stationary excitation state in terms of dynamical state level regardless of allosteric effects (Fig. 2–a)[16].

Effect of redox state (NADH/NAD$^+$ ratio, i.e., pyridine nucleotide potential) on the GF

The redox state expressed as NADH/NAD$^+$ was maintained at definite levels by the method shown in Fig. 3.

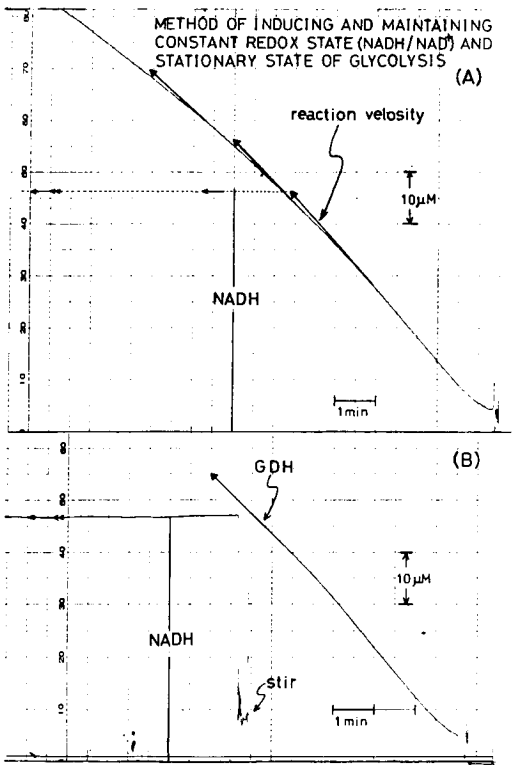

Fig. 3 As it is explained in Fig. 1, this is an example of realization of a constant redox state which is induced by the addition of glycerol-1-phosphate (-glycerophosphate) dehydrogenase. The rate of glycolysis can be estimated by tangent, i.e. reaction velocity along the progress curve (see the note in Table 5 and Fig. 1 about the coincidence between NADH formation defined GF in this expt. and GF, i.e. the rate of glycolysis estimated by the rate of pyruvate formation).

When it is necessary to carry out glycolysis under a constant NADH:NAD$^+$ ratio, an approproate amount of GDH must be added to the reconstructed system at the instant when the requested redox state is realized on the recording chart as shown in the case of (B).

Experimental conditions were the same as the standard system except that 0.11 mM of NAD$^+$ were used.

$\frac{NADH}{NAD} \times 10^2$	Velocity of Pyruvate Formation (observed) ($\mu M/min$)	G.F. ($\mu M/min$)	Pyruvate Formation (μM)
1.8	17.0	16.3	681
4.1	10.9	10.3	435
4.6	9.3	9.7	372
6.9	8.1	8.2	325
9.3	7.9	6.9	315
11.7	6.9	5.7	278
$\simeq 0$	17.8	16.9	713

Table 5. Effect of pyridine nucleotide on GF and comparison of rates of pyruvate formation with GF

There are respective rates of intermediate fluxes through glycolytic enzymes which may trap intermediate, since the amounts of glycolytic intermediates in the pathway are determined according to the equilibrium constants of the reaction catalyzed by enzymes. However, once a substrate, for instance glucose, is charged on the pathway and a steady state equilibrium is set up, the net fluxes of intermediates through the enzymes are usually identical*).

The values of the second column in this Table which is the rate of pyruvate formation are very close to the third column which represents GF observed as the rate of NADH generation.

Experimental conditions were the same as standard system except that the concentration of NAD^+ was decided to be low enough to set NADH/NAD^+ at an arbitrary ratio which must be observed on a recording paper as is explained in the note of Fig. 3; 0.1mM and 0.2mM were added to the system considering molar extinction coefficient of NADH at 340 nm.

*) Elmonl Coe: Nucleotide and glycolytic rate. Biochim. Biophys. Acta. 118, 495–511, 1966.

Table 5 shows GF corresponding to various levels of redox state and the rates of pyruvate formation.

The results indicate that GF is dependent upon the NADH/NAD^+ ratio and that the GF was significantly suppressed with the increase of NADH/NAD^+ ratio. A plot for the pyruvate production against NADH/NAD^+ ratio is shown in Fig. 2–b. There are two deflexions around 2.0 and 5.0 which show inverse sigmoidal curve. This would suggest that GF is controlled by the mechanism of allosteric activation on GAPDH by NAD^+ . Moreover, although simultaneous presence of stationary NADH $\rightleftharpoons$ NAD^+ coupling and allosteric effect by NAD^+ makes the elementary process cooperated with GAPDH more complex, the inverse sigmoidal curve suggests the conformational changes of GAPDH induced by NAD^+ [19].

Effect of NAD^+ level on the GF control.

Fig. 4 shows the time course of NAD^+ reduction which corresponds to GF at the various levels of NAD^+. The rate of NAD^+ reduction increased in accordance with concentration of NAD^+.

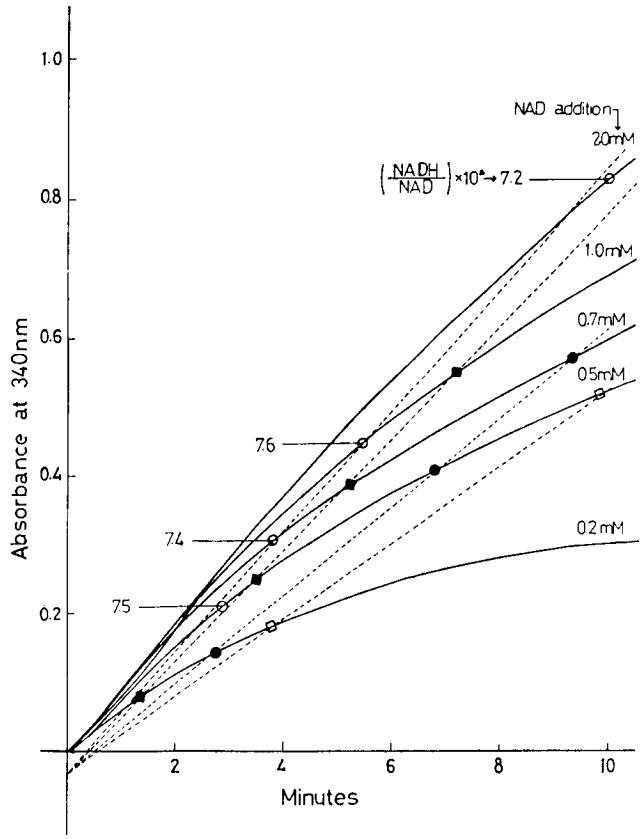

Fig. 4 Correlation between NADH:NAD^+ ratios and GF.
The progress curves were drawn by tracing the line based on the recorded charts. The plots of broken lines pass through the points of identical tangent value which is dependent upon the time and the amount of NAD^+ addition. Conditions were the same as Fig. 3 except that varied amount of NAD^+ were used.

Moreover, it can by shown another type of control linked to NADH/NAD^+ ratio; that is, the plots of the points of the same rate corresponding to the different NAD^+ addition revealed linear relationship each other and the broken lines come together to a point on the ordinate. On one hand, the points which have the same rate of NAD^+ reduction revealed almost identical NADH/NAD^+ ratio.

These results descrived above suggest that the redox state has an important role in the rate control of GF. This would also indicate that the redox state is significantly related with time factors of glycolysis not only for steady state or quasi-steady state but also for the determination of frequency and amplitude of oscillatory state in terms of retardation effect by NAD^+ on the decrease of NADH formation.

The effects of enzyme concentrations on GF.

Table 6 lists GF corresponding to the different enzymic profiles which were reconstituted by fixing constituent enzyme activities at 1.0 unit except for the enzyme used as a variable one. In

	Glycolytic Flux (μM/min) Unit of Enzyme						Normalized effect Unit of Enzyme					
	1	3	5	8	10	15	1	3	5	8	10	15
PFK	11.9	18.2	21.0	20.6	19.5	18.2	1.0	1.5	1.8	1.7	1.6	1.5
[ALD TIM]	14.1	12.5	12.1	10.6	12.1	—	1.0	0.89	0.86	0.75	0.86	—
[GAPDH PGK]	12.7	17.8	20.2	21.0	21.6	22.4	1.0	1.4	1.6	1.7	1.7	1.8
GAPDH	12.5	17.7	20.5	20.8	22.4	22.9	1.0	1.4	1.6	1.7	1.8	1.8
PGK	13.5	13.6	13.9	10.8	—	13.3	1.0	1.0	1.0	0.8	—	1.0
[PGM ENO]	16.1	15.3	14.5	13.5	14.5	—	1.0	0.95	0.90	0.84	0.90	—
PK	12.5	—	11.7	10.4	9.4	8.2	1.0	—	0.94	0.83	0.75	0.66

Table 6. Effect of constituent enzyme proportions on GF
The activities of enzymes added to the reaction mixture were 1.0 unit except for the enzyme as a variable one. Other conditions in this experiment were the same as standard system. Right block of this table shows the normalized effect which represents the values calculated by dividing the GF by the GF shown in first column of the left block of this table. In the several cases in which data are not available the reconstruction was not carried out successfully because the enzymes accidentally lost their activities during overnight dialysis before use.

order to estimate the effects of each constituent enzyme on GF, the activities of variable enzymes were varied from 1.0 unit to 15 units. The significant responses to the changes of variable enzymes were observed only when PFK or GAPDH was varied, and the stimulative effects on the GF was 50% and 80%, respectively. It is interesting that the increment in GAPDH affected the GF as strong as the important coupled enzymes, i.e. GAPDH and PGK which catalyze substrate level phosphorylation in glycolysis (Fig.1). One the contrary the increment of PGK did not affect the GF at all. This might indicate that these enzymes operate as a coupled enzyme system for substrate level phosphorylation, but they functionally operate as different enzymes for GF control, i.e., GAPDH is independent rate-controlling enzyme in the flux control. The fact that the enzymes which affected GF strongly were GAPDH and PFK suggests the characteristic roles of so-called "constant proportion group"[20] of goycolytic enzymes and allosteric enzymes.

Control characteristics of structural control variables

The necessary information for the analysis of characteristic behavior of a pathway would be at least the properties of constituent enzymes, particularly Km for substrates and for coenzymes and so on. However, if we want to investigate, furthermore, the dynamical behavior of the pathway, it is necessary to estimate quantitatively characteristic effects of the constituents on the pathway. One of the methods for estimation of the dynamical properties of the pathway is to obtain such parameters as control strength and sensitivity coefficient according to modern control theory.

SCV	$\frac{\Delta F_G}{\Delta a_i}$	$\frac{a}{F_G}\frac{\Delta F_G}{\Delta a}\times 10$
NAD	7.7	4.2
AMP	45.0	80.0
ADP	7.0	1.2
ATP	16.0	1.6
Pi	15.0	62.0
Mg	8.8	15.0
K	16.0	0.6
PFK	6.3	5.3
ALD TIM	-1.5	-1.1
GAPDH PGK	5.1	4.0
GAPDH	5.2	4.2
PGK	0.1	0.1
PGM ENO	-0.3	-0.2
PK	-0.4	-0.3

Table 7. Estimated steady state control characteristics of structural control variables (SCV)
The second column represents control strength and the third column indicates sensitivity coefficient times ten. The factor 10 was multiplied just for the purpose of convenience to compare with control strength. The calculations were carried out based on the data in the tables using equations shown in the first row of this table.

As it is summarized in Table 7, most of the significant dynamical characteristics of the members of glycolysis are reflected in the control parameters. For example, AMP showed the largest control strength and sensitivity coefficient. This result inducates an important feature that AMP is the most effective allosteric activator of PFK as well as influencing the enzyme as a deinhibitor of ATP[17)18)]. Inorganic phosphate which has the similar effect on PFK as AMP also showed fairly high control strength and sensitivity coefficient[17)18)]. On the contrary ATP which is a representative allosteric inhibitor of PFK had very low sensitivity coefficient, while it showed rather large control strength. This contradictory characteric may be interpreted by the fact they ATP play a role in the promotion of glycolysis as a substrate and on the other hand it affects also phosphofructokinase as allosteric inhibitor.

Potassium showed fairly large control strength. On the other hand rather small sensitivity coefficient was observed. These results suggest a characteristic of potassium that it plays a role as an activator of PK. However, it does not have allosteric effect on the enzyme. Magnesium showed intermediate control strength and revealed rather high sensitivity coefficient. This result may be

an indirect effect on ATP activation by the formation of active ATP-Mg^{++} complex. Control characteristics of the enzymes were also reflected in the dynamical parameters, i.e., only the allosteric enzymes revealed relatively high levels of control strength and sensitivity coefficient.

DISCUSSION

The present studies focus on characterizing the influence on GF by the whole constituent members of glycolysis. In other words, the experiments and results described above are expected to be useful for the estimation of the factors that affect significantly the capacity of glycolysis in vivo and in vitro.

First of all it should be pointed out that glycolysis has an important role on energy supply to the cells independent from mitochondria, especially in anaerobic state such as the conditions of heavy muscular activity and of fermentation in micro-organism. Control properties of glycolysis as a multi-enzyme system are now well documented by many reports in terms of allosteric effects, particularly related to the oscillatory control mechanism. On the other hand, many problems confront us; our knowledge on the quantitative control characters by the members of glycolysis is far from complete, especially in understanding the response of GF to the enzyme profiles and control elements except for allosteric effectors.

The steady state analysis of glycolysis based on other new concepts has been carried out by authors[21] and by Rapaport[22)25)]. Since the relative strength of the influence by the constituents of glycolysis is necessary information regarding the dynamical character of the pathway, they have tried to obtain the dynamical parameters of glycolysis by the estimation of "control strength and sensitivity coefficient" of glycolytic constituents in terms of flux control.

Enzyme profiles and allosterism.

The glycolytic rate is, of course, to some extent dependent upon the glucose concentration of the medium. However, for example, maximal rates, determined by isotopic methods, have been observed at less than 0.25 mM-glucose with human erythrocyte[23]. Above the commonly used glucose concentration of 7 – 10mM the glycolytic rate is independent of the glucose concentration. Therefore, under the conditions of this experiment GF is dependent upon the members of glycolysis except for glucose. On the other hand, Michaelis constant (Km) of the enzymes is thought to be one of the rate-controlling factors of glycolysis.

As far as the glycolytic rate is concerned, Km of glycolytic enzymes can never be saturated by the glycolytic intermediates, because the Km of the listed enzymes in this report is much higher than glycolytic rate itself[13]. Nevertheless, glycolysis is in fact strictly controlled in vivo and in vitro by many factors and conditions such as the concentration of intermediates. The overall capacity of glycolysis under steady state, as a result, must be primarily determined by the amount of enzymes, i.e. enzyme profiles, as well as the characteristic properties of enzymes such as allosteric interaction which realizes the feedback and feedforward control in the pathway.

It is obvious from the results in the present studies that GF is dependent upon the concentration of glycolytic enzymes which are classified into two major groups: the one is the high control strength group, i.e., PFK, GAPDH, and GAPDH-PGK couple, and the other is the low control strength group, i.e., ALD-TIM, PGM-ENO, PGK and PK as it is shown in Fig. 5 and Table 6. The important feature

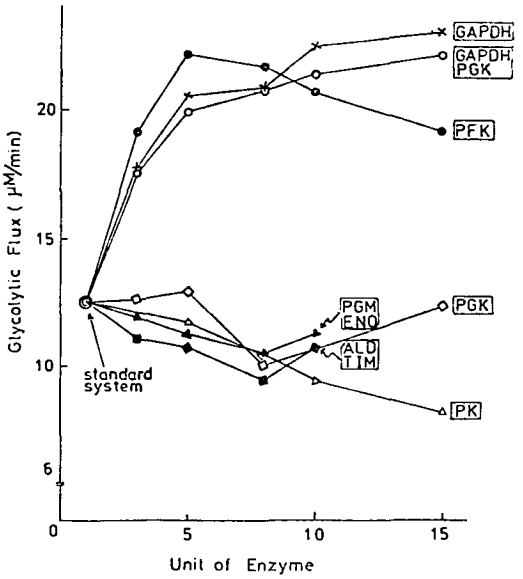

Fig. 5 Effect of constituent enzyme proportions on GF.
The plots are drawn based on the data in Table 6.

of the first group is the allosteric property which is realized by their conformational change induced by allosteric effectors such as ATP, ADP, AMP, NAD^+ and Pi. So-called allosteric interactions have Boolean characteristic, which may be expressed as "Flip-Flop" or "All or None" in the change of enzyme activities under the influence of allosteric effectors. As we have already described in the results, allosteric property of the enzymes seems to operate in the control of the pathway, particulary in the case of AMP and NAD^+ addition. These type of control may be found in Table 3 and Fig. 2–b.

According to Bücher and von Fellenberg[20)24)] glycolytic enzymes in tissues and cells are classified into two functionally related groups, i.e. so-called constant proportion group (TIM, PGK, PGM, ENO, GAPDH) which has definite proportion with respect to their activities and the biuret value of protein concentration among many kinds of tissues and cells, and the other group of enzymes which vary their amounts depending upon the conditions.

The results in the present studies indicate that the enzymes of constant proportion group do not necessarily play a significant role in controlling glycolysis except for GAPDH. Many authors report that GAPDH are present extraordinarily large amount in the cells, especially in skeletal muscles which have very high capacities of glycolysis. Of special interest is to indicate that GAPDH showed a very significant contribution on the enhancement of GF in the reconstructed system as well as PFK which does not belong to constant proportion group. On the contrary, PK which belongs to non-constant proportion group, plays an important role in glycolysis by generation of ATP whereas it has negative value of control strength and of sensitivity coefficient as shown in Table 6. The negative correlations between the amount of enzymes added to the system and their enhancement effect on the GF except for the case of PFK and GAPDH suggests the importance of allosterism of the enzymes in the control of glycolysis.

Coenzyme potential and regulation of GF

The frequent participation of adenine nucleotide system in phosphate transfer reactions and of pyridine nucleotide system in hydrogen transfer reactions makes these systems extremely important for multi-cite control in metabolic pathway such as glycolysis and mitochondrial metabolism.

The coenzyme potential in the cytosol is deeply correlated with the mitochondrial metabolism in vivo and in vitro. In vivo system the adenine nucleotide potential is determined by the coupling of glycolysis and oxidative phosphorylation. On the other hand, anaerobic condition in which glycolysis plays the main role in energy metabolism simplifies the control patterns of the pathway, since glycolytic pathway is dependent neither upon the hierarchy of Pasteur effect nor Crabtree effect[8)10)]. Therefore, the information such as control character of coupled coenzyme system obtained from the experiments on the reconstructed system of glycolysis will be useful for the interpretation of more complex phenomena of metabolic pathway.

It is pointed out that the coenzyme potentials are regulated by the following reaction.

$$GAP + NAD^+ + ADP + Pi \rightleftarrows 3PG + NADH + ATP$$

The capacity of GAPDH and PGK in the liver, muscle and many other tissues is very high, and the equlibrium relation defined by the following equation is considered to be a "master regulator" in the regulation of cytosolic phosphate potential and redox state which we have discussed in this paper.

$$\frac{[NAD^+]}{[NADH]} = K\frac{[3PG]}{[GAP]} \quad X \quad \frac{[ATP]}{[ATP]\ [Pi]}$$

However, the results we have obtained in the present experiment so far did not substantiate the theory of control mechanism described above, and on the contrary GAPDH and PGK seemed to influence on glycolysis independently each other.

Usage of control strength and sensitivity coefficient as the dynamical state parameter

The analysis of linear steady-state of enzymatic chain reaction such as glycolysis has been often examined in erythrocyte, muscle extract, yeast and other partially reconstructed system[8)11)22)25)]. However, it is necessary to examine an analysis in totally reconstructed system of glycolysis, because control characteristics of the structural control variables should be "eigentlich" property decided by their molecular structures and interaction with the environment. By conditioning their environment as simple as possible it will be able to get access to real evaluation of the parameters. Thereafter, the application to cells or tissues will be qualified by considering discrepancy between living system and model system as a perturbation.

Data on control strength estimated in case of erythrocyte were much smaller than those of the reconstructed system. For example, control strength of HK-PFK system, PFK and PK was 1.37, 0.31 and 0.04 respectively[22)25)]. The large discrepancy of control strength between reconstructed system and erythrocyte in the case of PFK may be ascribed to manifestation of strong allosteric interaction in the high purity of enzyme and to extremely complex perturbation even in such a simple system as red cells. By comparison of the data mentioned above with those of reconstructed system the relative correlation among enzymes in the control of pathway will be established and the possibility of application of parameters obtained in the experiments of reconstructed system will be feasible to the intact living system.

SUMMARY

Glycolytic system was reconstructed with purified enzymes, inorganic ions and nucleotides for the analysis of the steady-state characteristics of the glycolytic pathway. Each amount of the enzymes used was 1.0 international unit except for the enzymes as a variable one. Amounts of the constituents of glycolysis were varied so as to obtain the control parameters such as control strength and sensitivity coefficients which characterize the dynamical properties of the pathway. GF was strongly enhanced in the presence of 0.1mM or more of AMP, and it was also enhanced by ADP and ATP until the concentration of about 2mM. Effect of ATP/ADP ratio showed most efficient effect on GF at one to one ratio. Constant level of redox state was maintained by the addition of appropriate amounts of GDH. And moreover, arbitrary steady-state of glycolysis was produced by maintaining $NADH/NAD^+$ ratio at constant level. Time for attaining definite GF depended direct-proportionally on NAD^+ level. PFK and GAPDH are known to effect on GF as the allosteric enzymes of the pathway. These enzymes had large control parameters showing the effective enhancement of GF. PGK had small values of parameters, but had large values with cooperation of GAPDH. On one hand GAPDH alone strongly affected GF. This may contradict the fact that these enzymes facilitate the ATP formation at substrate level to effect GF because they locate on a sequence of process of the pathway. Rapaport reported on the steady-state analysis of glycolysis in erythrocytes. The results obtained in the reconstructed system were compared with those by Rapaport and discussed.

REFERENCES

1) Krebs, H.A.: Control of metabolic process. Endeavour, 125–132, 1957.
2) Ghosh. A., Chance, B.: Oscillation of glycolytic intermediates in yeast cells. Biochem. Biopys. Res. Comm. 16, 174–181, 1964.
3) Higgins, J.: A chemical mechanism for oscillation of glycolytic intermediates in yeast cells. Biochemistry 51, 989–994, 1964.
4) Betz, A., Chance, B.: Phase relationship of glycolytic intermediates in yeast cells with oscillatory metabolic control. Arch. Biochem. & Biophys. 109, 585–594, 1965.
5) Frenkel, B.: Reduced diphosphopyridine nucleotide oscillations in cell-free extract from beef heart. Arch. Biochem. Biophys. 115, 112–121, 1966.
6) Sel'kov, E.E.: Self-oscillations in glycolysis. Eur. J. Biochem. 4, 79–86, 1968.
7) Chance, B., Pye, E.K., Ghosh. A., Hess, B.: Many titles under the heading "Glycolytic oscillations" in "Biological and biochemical oscillation". Academic press, N.Y. 127–300, 1973.
8) Gatt, S., Racker, E.: Regulatory mechanism in carbohydrate metabolism. J. Biol. Chem. 234, 1015–1023, 1959.
9) Wu, R., Racker, E.: Regulatory mechanism in carbohydrate mechanism. J. Biol. Chem. 234, 1029–1035, 1959.
10) Wu, R., Racker, E.: Regulatory mechanism in carbohydrate metabolism. J. Biol. Chem. 234, 1036–1041, 1959.
11) Uyeda, K., Racker, E.: Coordinated stimulation of hexokinase and phosphofructokinase by phosphate in a reconstructed system of glycolysis. in "Control of energy metabolism" ed. Chance, B., estabrook, R.W., Williamson, J.R. Academic Press, N.Y., p.127–137, 1966.

12) Krebs, H.A.: Regulation of the concentration of low molecular constituent in compartments., in "Microenvironments and metabolic compartmentation" ed. Srere, P.A., Estabrook, R.W. Academic press, N.Y. p.3–16, 1978.
13) Barman, T.E.: Enzyme Handbook, vol. 1 and vol. 2, Springer verlag, Berlin Heiderberg, New York, 1969.
14) Bergmeyer, H.U.: Methods of enzymatic analysis, 2nd ed. Acamedic press, N.Y. 1965.
15) Bruns, J.A.: Steady state of general multi-enzyme net works and their associated properties. computational approaches. FEBS letters, 2, supplement, S30–S33. 1969
16) Higgins, J.: Dynamics and control in cellular reaction., in "Control of energy metabolism" ed. Chance, B., Estabrook, R.W., Williamson, J.R. Academic press, N.Y. p.13–46, 1966.
17) Lowry, O.H., Passonneau, J.V.: Kinetic evidence for multiple binding site on phosphofructokinase. J. Biol. Chem. 241, 2268–2279, 1966.
18) Passonneau, J.V., Lowry, O.H.: The role of phosphofructokinase in metabolic regulation., in "Advances in enzyme regulation" vol. 2, p.265–274, 1964.
19) Koshland, Jr. D.E., Conway, J.A. Kirtley, M.E.: Conformational changes and the mechanism of action of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase., in "Regulation of enzyme activity and allosteric interactions", FEBS symposium 3–7 July 1967. Academic press, N.Y. p.131–143, 1968.
20) Fellenberg, R.V., Eppenberger, H., Richterich, R., Aebi, H.: Das glykolytische Enzymmuster von leber, Niere, Skeletmuskel Herjmuskel und Grosshirn bei Ratte und Maus. Biochem. Z. 336, 334–350, 1962.
21) Sakurai, T., Shinjo,S., Arashiro, J., Asato, R.: Studies on the mechanism of the steady state control of reconstructed glycolytic pathway. J. physiol. Soc. Japan 36, 412, 1974.
22) Rapaport, T.A., Heinrich, R., Jacobash, G., Rapaport, S.: A linear steady state treatment of enzymatic chains. Eur. J. Biochem. 42, 107–120, 1974.
23) Garby, L., Verdier, C.H.: Glucose metabolism in normal erythrocytes. Scand. J. Haemat. 1, 150–167, 1964.
24) Hess, B., Brand, K.: Enzyme and metabolite profiles., in "control of energy metabolism", ed. Chance, B., Estabrook, R.W., Williamson, J.R. Academic press, N.Y., p.111–122, 1966.
25) Heinrich, R., Rapaport, T.A.: The regulatory principles of the glycolysis of erythrocytes in vivo and in vitro., in "Mathematical model of metabolic regulation"ed. Kelet,T., Lkatos, S. Akademiac Kiado. Budapest, p.173–212, 1976.