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# The Relationships between Substrates and Enzymes of Glycolysis in Brain*

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In the preceding paper (1) are recorded the concentrations in mouse brain of the substrates and cofactors of the Embden-Meyerhof pathway, and the changes in concentration when the glycolytic rate is increased many fold by sudden ischemia (decapitation). In this paper, the concentrations found are examined in relationship to the activity and kinetic properties of each enzyme concerned and the glycolytic flux.

To make this possible, the activities of all enzymes of glycolysis in mouse brain except triosephosphate isomerase were measured under conditions designed to simulate the pH and ionic environment of the cell, and abbreviated kinetic studies were made of each enzyme under these same conditions.

Many of the steps involved are reversible reactions with low $\Delta F$ values. Bücher and Klingenberg (2) and others from the same laboratory (3, 4) have concluded that in liver and muscle many steps of glycolysis and of related systems are maintained close to equilibrium even during increased activity. Hess (5) has come to similar conclusions in regard to ascites tumor cells. For these reasons, and because of a major discrepancy between observed and expected substrate levels at the aldolose step, some of the equilibria have been redetermined under the simulated conditions *in vivo*.

The combined analytical and kinetic information makes it seem likely that along the glycolytic pathway in mouse brain (*a*) no step is limited by the amount of enzyme present; (*b*) equilibrium is approximated at five or possibly six steps even during maximal glycolysis; (*c*) one step (phosphoglycerate kinase) never reaches equilibrium whereas three others (counting $\alpha$-glycerophosphate dehydrogenase) are not maintained at equilibrium during rapid glycolysis; and (*d*) the hexokinase and phosphofructokinase steps are the only points at which there is absolute control of glycolytic flux. Those steps which do not control flux, but which are not rapid enough to maintain equilibrium, influence the system to the extent that they raise the levels of substrates further upstream. They serve the function of a dam but not of a valve.

## EXPERIMENTAL PROCEDURE

All enzyme measurements and kinetic studies were carried out in 1 ml at pH 7 to 7.2 in 20 mM imidazole buffer containing 150 mM potassium acetate, 5 mM $MgCl_2$, 5 mM $K_2HPO_4$, and 0.02% bovine plasma albumin. Other additions were made as necessary for the study of individual enzymes (Table I).

With one or two minor exceptions, all measurements were made directly in a fluorometer by following the increase or decrease in DPNH or the increase in TPNH. Auxiliary purified enzymes were used as needed. The sensitivity of fluorometry permitted the study, when necessary, of activities with substrate levels as low as $10^{-7}$ M.

It is probable that some of the enzymes activities are represented by several molecular species. Therefore, for the present purpose, it was felt desirable to conduct the kinetic studies with unfractionated whole brain homogenate. The high sensitivity available made it possible to employ very high tissue dilutions for the kinetic studies. This helped to avoid disturbing side reactions from other enzymes present.

Most of the procedures were adapted to the fluorometer (Table I) from spectrophotometric assays developed by others (7). Specific mention should be made of the study of Wu and Racker (8), in which spectrophotometric assays for all of the enzymes of glycolysis in ascites tumor cells are described, and that of Beck (9), who measured most of these enzymes in white blood cells as well as the kinetic constants.

Most of the kinetic studies were performed at 26–29°, but a few measurements were also made for each enzyme at 38° at two substrate levels: one high enough to approach enzyme saturation and another in the range found in brain *in vivo*.

The substrates were obtained from California Corporation for Biochemical Research (dihydroxyacetone-P, glyceraldehyde-P), Boehringer and Sons through California Corporation for Biochemical Research (fructose diphosphate, 3-P-glycerate, 2-P-glycerate, P-enolpyruvate), and Sigma Chemical Company, (glucose-6-P, pyruvate, ATP, ADP, AMP, DPN+, DPNH, TPN+). The fructose-6-P was prepared from fructose with brain hexokinase.

## RESULTS

*Enzyme Activities*—In terms of maximal velocities (Table II), on the main pathway the least active enzyme per unit of tissue weight is aldolase, with hexokinase and P-fructokinase only slightly more active. However, even aldolase activity is 2 to 3 times greater than the maximal glycolytic flux observed in brain, so that no enzyme of the series is necessarily limiting in the absolute sense. P-Glycerate kinase has by far the greatest activity in brain among the enzymes of the glycolytic series, as Wu and Racker found true for ascites tumor cells (8). The

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TABLE I

*Conditions for kinetic measurements*

The basic composition of the medium is given in the text. Auxiliary enzymes were from muscle except for glucose-6-P dehydrogenase, which was from yeast. All were purchased from Boehringer and Sons through Calbiochem. Brain homogenate was added in sufficient quantity to give rates of 0.2 to 2 μM per minute. The homogenates were prepared in 0.04 M phosphate buffer, pH 7, and diluted in buffer with 0.02% added bovine plasma albumin. All enzymes were measured in the direction of glucose to lactate or glycero-P, except that P-glucoisomerase was measured in both directions.

| Enzyme | Auxiliary enzymes | Other additions (except substrates) |
|---|---|---|
| Hexokinase | Glucose-6-P dehydrogenase, 1 μg/ml[a] | $TPN^+$, 0.02 mM |
| Aldolase | Triose-P isomerase, 2.5 μg/ml; glycero-P dehydrogenase, 2.5 μg/ml | DPNH, 0.008 mM |
| P-Fructokinase | As above, plus aldolase, 5 μg/ml | DPNH, 0.008 mM |
| P-Glucoisomerase, forward | As above plus P-fructokinase[b] | DPNH, 0.008 mM; ATP, 3 mM; AMP, 0.06 mM |
| P-Glucoisomerase, reverse | Glucose-6-P dehydrogenase, 1 μg/ml[a] | $TPN^+$, 0.02 mM |
| Glycero-P dehydrogenase | None | None |
| Glyceraldehyde-P dehydrogenase | None | Mercaptoethanol, 5 mM |
| P-Glycerate kinase | Glyceraldehyde-P dehydrogenase, 2 μg/ml | Mercaptoethanol, 5 mM, plus $DPN^+$[c] and glyceraldehyde-P[c] |
| Pyruvate kinase | Lactic dehydrogenase, 2.5 μg/ml | DPNH, 0.008 mM |
| Enolase | Lactic dehydrogenase, 2.5 μg/ml; pyruvate kinase, 5 μg/ml | DPNH, 0.008 mM |
| P-Glycerate mutase | As above plus enolase, 0.8 μg/ml | DPNH, 0.008 mM; 2,3-diphosphoglycerate, 0.002 mM |
| Lactic dehydrogenase | None | None |

[a] Equivalent to about 0.3 μg of crystalline enzyme per ml (6).

[b] Sufficient partially purified enzyme from muscle was used to provide maximal activity of 10 mM per hour.

[c] These were varied as needed to provide the different levels of 1,3-diphosphoglycerate.

correspondence in absolute terms between their data for ascites tumor cells, Wu's values for HeLa cells (10), and these data for brain is surprisingly close.

The glycolytic enzyme activities in the brains of 10-day-old mice averaged 66% of adult values. The range was narrow, 55% to 74%, except for lactic dehydrogenase, which was 92% of the adult value. The increases of glyceraldehyde-P and glycero-P dehydrogenases with age were previously observed in rat brain by Laatsch (11). He found that glycero-P dehydrogenase increases much more than glyceraldehyde-P dehydrogenase during the first few days of life. The effect of age on lactic dehydrogenase in rat brain has been reported earlier (12).

The present data are based on wet weight, because this was the basis for the substrate values. Since protein concentration increases between 10 days of age and maturity, the increases in enzyme activity would be considerably less on a protein basis.

*Kinetic Constants*—Because of the mentioned possibility that whole brain might contain several species of protein with the same enzyme activity, the kinetic parameters (Table III) may be regarded as attributes of the tissue as a whole rather than as properties necessarily of pure molecular species. Particular stress is placed on kinetic properties in the range of substrate concentrations encountered in brain.

The Michaelis constant and temperature coefficient for each enzyme are recorded together with a value which appears most significant as a determinant of substrate levels in the cell, the ratio of $V_{max}$ to $K_s$. This ratio has the properties of a first order rate constant and will be denoted as $k_a$ or the "tissue first order rate constant." If an enzyme and its substrate are evenly distributed in a tissue, $k_a$ is the rate constant for that substrate when its concentration, $(A)$, is low compared to $K_s$. In this case, in the absence of back-reaction, the concentration of a substrate would be determined by the relationship $(A) = \text{flux}/k_a$.

It is of interest to compare Michaelis constants with observed substrate levels (Table III). For the six enzymes which convert glyceraldehyde-P to lactate, no substrate level rose higher during peak glycolysis than 70% of the $K_s$. Of the steps preceding glyceraldehyde-P dehydrogenase, for all but P-fructokinase, which is a special case, the substrate level may rise above $K_s$.

Specific comments about certain of the enzymes are as follows.

*Hexokinase* (*Fig. 1*)—The $K_s$ for glucose was found to be almost independent of ATP concentration down to 16 μM, but $K_{ATP}$ was increased by bringing glucose to very low levels (Table III). The Michaelis constants are in the range of those obtained with purified calf brain hexokinase by Fromm and Zewe (15).

The phosphorylation of glucose, like that of fructose-6-P, seems to be a controlling step in glycolysis; therefore, hexokinase was studied to see if it shares any of the peculiar kinetic properties of P-fructokinase (see below). No resemblance was seen. Levels of ATP as high as 10 mM were not inhibitory even with low glucose levels. ADP, AMP, 3′,5′-cyclic AMP, fructose diphosphate, and $P_i$, all activators of P-fructokinase in the presence of high ATP, were tested at 1 mM concentrations with 3 mM and 0.3 mM ATP, combined with 5 mM and 0.01 mM glucose. Of these five compounds, the last three were inactive, and ADP and AMP inhibited 30 to 50%. Glucose-6-P was studied extensively because of its well known inhibitory effect on mammalian hexokinase (16, 17).[1] It was observed that a plot of inhibitor

[1] The assays for hexokinase activity in the presence of glucose-6-P were conducted by measuring glucose disappearance as follows. In a first step, P-glucoisomerase was included but no $TPN^+$ or glucose-6-P dehydrogenase. The reaction was terminated with heat, and to each sample was added an equal volume of 0.1 M Tris, pH 8, containing excess $TPN^+$ plus glucose-6-P dehydrogenase and P-glucoisomerase. After oxidation of the hexose monophosphates was complete, a suitable aliquot was added to 1 ml of 0.05 M Tris, pH 8, and the TPNH fluorescence was measured. This gave the sum of glucose-6-P and fructose-6-P present at the end of the first incubation. The concentration of glucose-6-P during incubation was taken as the average of the initial and final hexose monophosphate concentrations (the differ-

TABLE II

*Enzyme activities in mouse brain*

The amount of brain used for each assay (1 ml, total volume) varied from 12 $\mu$g in the case of P-glycerate kinase to 0.5 mg in the case of glycero-P dehydrogenase. The values for $V_{max}$ at 38° were calculated from the averages at 29–30° by taking into account both temperature coefficients and Michaelis constants. The weight and age of each mouse are given in the table along with the average age and weight for Mice 1 to 3 and 4 to 6.

| Enzyme | Assay temperature | Substrate concentration | | Enzyme activities | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | A[a] | B | Mouse 1 | Mouse 2 | Mouse 3 | Average | $V_{max}$ at 38° | Mouse 4 | Mouse 5 | Mouse 6 | Average | $V_{max}$ at 38° |
| | | | | 5.5 g | 7.0 g | 6.5 g | 6.3 g | | 23 g | 24 g | 22 g | 23 g | |
| | | | | 10 days | 10 days | 10 days | 10 days | | Adult | Adult | Adult | Adult | |
| | | *mM* | | *mmoles/kg/min* | | | | | *mmoles/kg/min* | | | | |
| Hexokinase | 29° | 1.0 | 0.3 | 6.1 | 5.8 | 6.4 | 6.1 | 15.2 | 11.2 | 11.9 | 10.0 | 11.0 | 27.5 |
| P-Glucoisomerase | 30 | 0.2[b] | | 36 | 39 | 37 | 37 | 154 | 57 | 57 | 52 | 55 | 230 |
| P-Fructokinase | 29 | 0.2 | 1.0 | 8.0 | 7.8 | 7.7 | 7.8 | 26.7 | 11.0 | 10.9 | 10.2 | 10.9 | 36.1 |
| Aldolase | 29.5 | 0.3 | | 3.0 | 3.3 | 3.5 | 3.3 | 7.6 | 5.5 | 5.1 | 5.0 | 5.2 | 12.1 |
| Glycero-P dehydrogenase | 30 | 0.8 | 0.015 | 1.05 | 1.34 | 1.17 | 1.18 | 1.93 | 2.3 | 2.2 | 2.4 | 2.3 | 3.8 |
| Glyceraldehyde-P dehydrogenase | 29.5 | 0.4 | 0.2 | 38 | 42 | 42 | 41 | 96 | 57 | 58 | 62 | 56 | 131 |
| P-Glycerate kinase | 29.5 | 0.015 | 0.4 | 105 | 110 | 121 | 112 | 750 | 187 | 160 | 164 | 170 | 1140 |
| P-Glycerate mutase | 29.5 | 0.2 | | 26 | 25 | 26 | 26 | 145 | 42 | 36 | 33 | 39 | 218 |
| Enolase | 29.5 | 0.5 | | 17 | 17 | 18 | 17 | 36 | 32 | 30 | 28 | 30 | 62 |
| Pyruvate kinase | 28.5 | 0.6 | 1.6 | 64 | 69 | 67 | 67 | 95 | 120 | 122 | 111 | 118 | 165 |
| Lactic dehydrogenase | 28.5 | 1.0 | 0.02 | 51 | 60 | 53 | 55 | 129 | 65 | 56 | 56 | 59 | 140 |

[a] Substrate A refers to hexose or triose, and Substrate B refers to ATP, ADP, $DPN^+$, or DPNH, as the case may be. For glyceraldehyde-P dehydrogenase, the $P_i$ level was 5 mM. For mutase, 0.003 mM 2,3-diphosphoglycerate was added.

[b] Fructose-6-P concentration. The activities of P-glucoisomerase for the six individual mice were measured in the direction of glucose-6-P formation, and are so recorded; but the average values for $V_{max}$ at 38° are calculated for the opposite direction.

TABLE III

*Kinetic constants*

The first Michaelis constant, $K_A$, is for the carbohydrate substrate; the second, $K_B$, is for the nucleotide substrate when applicable. $K_A$ was measured at 38° with the concentration of B indicated. $K_B$ was measured at 26–29° with the concentration of A indicated. As described in the text and footnotes, in a number of cases there are mutual effects of one substrate on the Michaelis constant of the other. $k_a = V_{max}/K_A$; it is the equivalent of a first order rate constant in the tissue for Substrate A (see text). Temperature coefficients are the fractional change per degree rise of temperature between 29° and 38° with Substrate A either well above the Michaelis constant, "High (A)," or well below, "Low (A)."

| Enzyme | $K_A$ | (B) | $K_B$ | (A) | $k_a$ | | Temperature coefficient | | $(A)_{max}/K_A$[a] |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Adult | 10-Day | High (A) | Low (A) | |
| | *µM* | | *µM* | | *min⁻¹* | | | | |
| Hexokinase | 40 | 3,000 | 130[b] | 5,000 | 690 | 380 | 1.059 | 1.049 | 65 |
| P-Glucoisomerase | 210 | | | | 1,100 | 732 | 1.077 | 1.013 | 1.1 |
| P-Fructokinase | 270 | 1,000 | 25 | 200 | 134 | 98 | 1.041 | 1.013 | 0.18 |
| Aldolase | 12[c] | | | | 1,010 | 630 | 1.098 | 1.114 | 18 |
| Glycero-P dehydrogenase | 37 | 15 | 2.2 | 100 | 103 | 52 | 1.056 | 1.047 | 1.6 |
| Glyceraldehyde-P dehydrogenase | 44 | 200 | 22 | 9[d] | 2,980 | 2,180 | 1.070 | 1.075 | 0.08 |
| P-Glycerate kinase[e] | 9 | 500 | 70 | 2 | 56,000 | 37,000 | 1.11 | 1.00 | <0.1 |
| P-Glycerate mutase | 240 | | | | 910 | 600 | 1.113 | 1.105 | 0.35 |
| Enolase | 33 | | | | 1,880 | 1,090 | 1.08 | 1.07 | 0.37 |
| Pyruvate kinase | 55 | 1,400 | 180 | 24 | 3,000 | 1,730 | 1.031 | 1.031 | 0.06 |
| Lactic dehydrogenase[f] | 140 | 18 | 2.8 | 110 | 1,000 | 920 | 1.063 | 1.028 | 0.7 |

[a] (A) in this case is the peak value for Substrate A among the several groups of mice. It was an initial value in the case of glucose, glucose-6-P, and fructose-6-P, and a value during maximal glycolysis in the case of the rest (see Table III of the preceding paper (1)).

[b] $K_{ATP}$ was increased 80% when glucose was lowered to 9 µM.

[c] At 27°, this value was 28 µM.

[d] ($P_i$) was 5 mM. The $K_s$ values for $P_i$ at 28° were 0.4 mM and 2.2 mM, respectively, with 9 and 440 µM glyceraldehyde-P (200 µM $DPN^+$); the $K_s$ for glyceraldehyde-P was reduced 4-fold by reducing $P_i$ to 0.5 mM.

[e] $K_{ATP}$ was increased to 700 µM when 1,3-diphosphoglycerate was raised to 15 µM. With 3 mM ATP and 0.16 mM 3-P-glycerate, the rate was inhibited 67% (0.5 mM ADP and 2.4 µM 1,3-diphosphoglycerate); 3 mM ATP alone inhibited 35%, but 1,3-diphosphoglycerate alone was not inhibitory.

[f] At 28°, with 2.5, 18, and 117 µM DPNH, Michaelis constants for pyruvate were 45, 82, and 96 µM, respectively. Conversely, with 16, 30, 110, and 300 mM pyruvate, values for $K_{DPNH}$ were 1.8, 2.6, 2.8, and 3.9 µM, respectively. These reciprocal effects are just the opposite of those found by Hakala, Glaid, and Schwert (13) for heart lactic dehydrogenase.

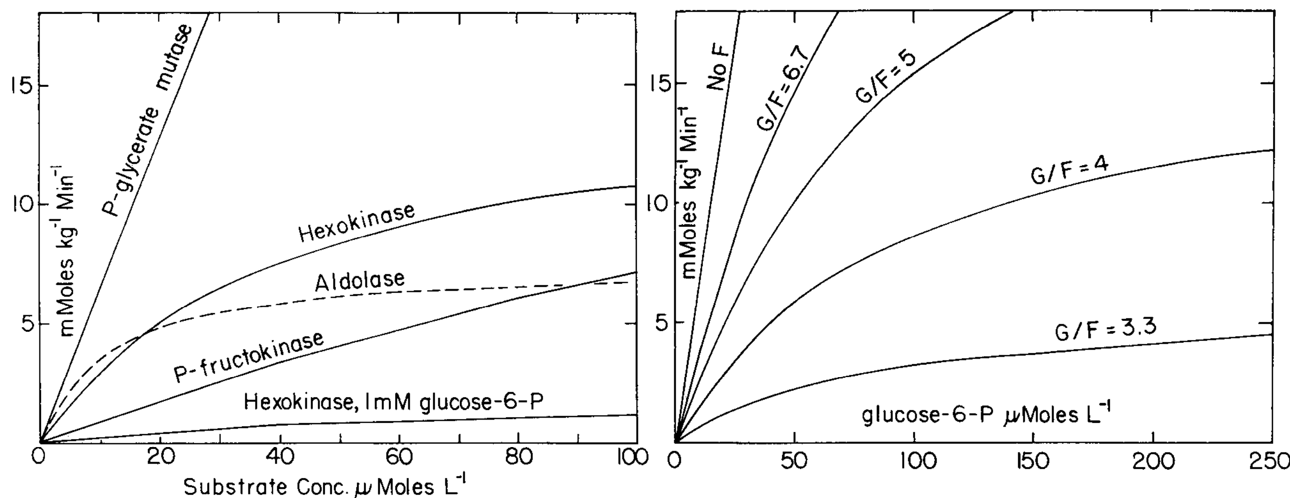


FIG. 1 (*left*). Hexokinase, P-fructokinase, aldolase, and P-glycerate mutase activity as a function of substrate concentrations. These are theoretical curves calculated for 10-day-old mouse brain at 38° from kinetic constants of Table III and average maximal activities. For adult mice, the ordinate is multiplied by 1.8, 1.35, 1.6, and 1.5 for the respective enzymes. Hexokinase velocities are calculated for 3 mM ATP levels; P-fructokinase activities are for 1 mM ATP and 5 mM $P_i$ levels.

FIG. 2 (*right*). Theoretical velocities of P-glucoisomerase of brain as a function of glucose-6-P concentration and various ratios of glucose-6-P (*G*) to fructose-6-P (*F*). The calculations are based on velocities in both directions measured under conditions that did not permit significant accumulation of product (Table I). From these observed rates, the velocities in the presence of product were calculated according to the following equation, which has previously been shown to be valid for muscle P-glucoisomerase (14). The equation takes into account both forward and back reactions as well as product inhibition.

$$v = \frac{V_G K_F(G) - V_F K_G(F)}{K_F(G) + K_G(F) + K_F K_G}$$

in which $V_G$ and $V_F$ are the maximal velocities in the absence of product in the forward and back directions respectively, $K_G$ and $K_F$ are the Michaelis constants for glucose-6-P and fructose-6-P, and (*G*) and (*F*) are the concentrations of the two substrates. For 10-day-old mouse brain, $V_G$ is 154 mmoles per kg per minute and $V_F$ is 93 mmoles per kg per minute, and for mouse brain enzymes, in general, $K_F$ is 42 μmoles per kg and $K_G$ is 210 μmoles per kg. By substituting these values for $K_G$ and $K_F$ and rearranging, the value of *R*, the ratio of (*G*) to (*F*), may be calculated.

$$R = \frac{3V_G + 5v}{V_G - v[1 + 210/(G)]}$$

The figure applies to 10-day-old mouse brain. For adults, the ordinate is multiplied by 1.5.

concentration against the reciprocal of velocity is not linear but rather curves in a manner which suggests that 2 molecules of glucose-6-P may be required for inhibition. The data gave a reasonable fit to a curve for the equation $V/v = 1 + 1/[K_2/(i) + K_1K_2/(i)^2]$, where $K_1 = 0.3$ mM, and $K_2 = 0.1$ mM. Nearly 90% inhibition was observed with 1 mM glucose-6-P. Only minor changes in glucose-6-P inhibition were observed with changes in pH (6.5 to 8.5), ionic strength, $Mg^{++}$, and $P_i$ concentrations. Nevertheless, it seems possible that some tissue factor may increase the glucose-6-P inhibition to the point where it could account for the observed control at this point.

*P-Glucoisomerase* (*Fig. 2*)—Under the conditions of these experiments, the kinetic constants are somewhat different from those observed earlier at pH 8 with lower ionic strength (14). As recorded in the legend to Fig. 2, the maximal velocity in the direction of fructose-6-P formation is actually faster than in the opposite direction even though equilibrium at 38° favors glucose-6-P 3:1 (14).

*P-Fructokinase*—The kinetics of this enzyme is exceedingly complicated and will be the subject of a separate report. Here it is only necessary to say that as originally discovered by Lardy and Parks (18), ATP at high levels is very inhibitory for P-fructokinase. This inhibition may be overcome by addition of levels of $P_i$, ADP, fructose-6-P, AMP, 3',5'-cyclic AMP, and fructose diphosphate, in this order of increasing potency (19). Combinations of several of these are particularly effective.

*Aldolase* (*Fig. 1*)—The $K_s$ for fructose diphosphate agrees well with that found by Richards and Rutter for the muscle enzyme (20). The combined effects of product inhibition and back-reaction were assessed by incubating fructose diphosphate at 100 μM and 500 μM concentration (*i.e.* the physiological range) with mouse brain diluted 1:12,500 and 1:3,000, respectively. Samples were withdrawn at 5-minute intervals and analyzed for dihydroxyacetone-P and glyceraldehyde-P. With both substrate levels, the rate of reaction fell to about half when the reaction had gone halfway to equilibrium. (The triose phosphates were in approximate equilibrium with each other at this time.) According to these results, if fructose diphosphate has free access to aldolase, flux/$V_{max}$ would approximate the fractional deviation of substrate and products from equilibrium. For example, if flux is one-fourth of $V_{max}$, substrates would be within 25% of equilibrium.

*Glyceraldehyde-P Dehydrogenase* (*Fig. 3*)—The $K_s$ for $P_i$ was increased sharply by raising glyceraldehyde-P, and, conversely, raising $P_i$ raised the $K_s$ for glyceraldehyde-P (Table III). These effects are in keeping with the mechanism proposed by Velick and Hayes (21). A practical consequence of this is that at the low levels of glyceraldehyde-P found in brain, $P_i$ concentration will have little effect on rate unless it falls below 0.5 mM (Fig. 3).

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ence was kept small) multiplied by 0.82, *i.e.* the ratio of glucose-6-P to total hexose phosphate at 25° (14). The remainder of the original sample was made 0.1 N in HCl to destroy the TPNH, and a suitable aliquot was added to 1 ml of 0.05 M Tris buffer, pH 8, containing 5 mM $MgCl_2$, 0.3 mM ATP, and 0.1 mM $TPN^+$. The glucose itself was now measured by the fluorescence produced on the addition of hexokinase and glucose-6-P dehydrogenase.

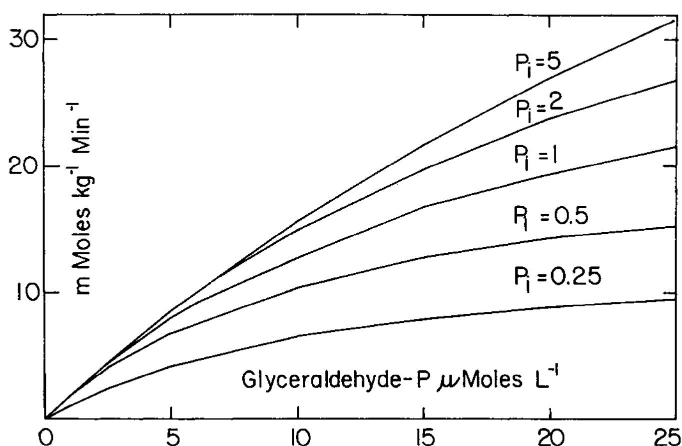



FIG. 3. Glyceraldehyde-P dehydrogenase activity as a function of substrate concentration at a number of levels of $P_i$. These are theoretical curves calculated for 10-day-old mouse brain at 38° from kinetic constants and average maximal activities (see the text). For adults, multiply by 1.37. The rates are for a 0.2 mM DPN+ level.

*P-Glycerate Kinase* (*Fig. 4*)—Of the enzymes of glycolysis, P-glycerate kinase has not only the highest maximal velocity but also the smallest Michaelis constant; consequently, $k_a$ for brain is very large (Table III), sufficient to give a substrate turnover time of about 1 millisecond in the adult. The kinetic data suggest a situation similar to that with glyceraldehyde-P dehydrogenase, in that an increase in 1,3-diphosphoglycerate causes a large increase in $K_{ADP}$. There is a similar practical consequence; at the very low substrates levels present in brain, rate should be independent of ADP concentration unless it falls far below observed levels. Combinations of ATP and 3-P-glycerate in the physiological range were moderately inhibitory, in part because of back-reaction and in part because of ATP inhibition (Table III). It will be noted that the maximal velocity has a high temperature coefficient, whereas with low substrate, the temperature coefficient is very small. This results in a high temperature coefficient for the Michaelis constant, and indicates a high temperature coefficient for $k_3$ but not for $k_2$ in the expression $K_s = (k_2 + k_3)/k_1$.

*P-Glycerate Mutase* (*Fig. 1*)—The substrate used was not free of 2,3-diphosphoglycerate, so that activity was found without added coenzyme. The velocity was tripled by addition of 3 μM coenzyme. Since 6 μM coenzyme did not further increase activity, the $K_s$ must be below 1 μM, which is much lower than that reported for the enzyme from muscle (22, 23). Studies with both brain homogenate and crystalline muscle enzyme suggest that requirements for coenzyme increase as substrate increases. The tissue concentration of the coenzyme formed in brain, about 20 μM (1), seems ample for full mutase activity.

*Enolase* (*Fig. 5*)—The $K_s$ for brain enolase is about one-third of that reported by Holt and Wold for muscle enolase (24) in a somewhat different medium (0.4 M KCl instead of 0.15 M potassium acetate).

*Pyruvate Kinase*—The $K_s$ values observed are close to those found by McQuate and Utter (25) and Reynard *et al.* (26) for muscle pyruvate kinase. In agreement with the former workers, the $K_s$ for each substrate is increased by high levels of the other. Reynard *et al.* did not see this effect, but they worked at a much more alkaline pH (8.5). The constants given (Table III) were obtained by using the intercepts of a Lineweaver-Burk plot (made with several levels of the second substrate) in a second reciprocal plot. ATP inhibition was observed. This appeared to be noncompetitive with either substrate, with a $K_i$ of about 1 mM. This differs from the muscle enzyme, which shows competition of ATP with both substrates (26). Of more significance

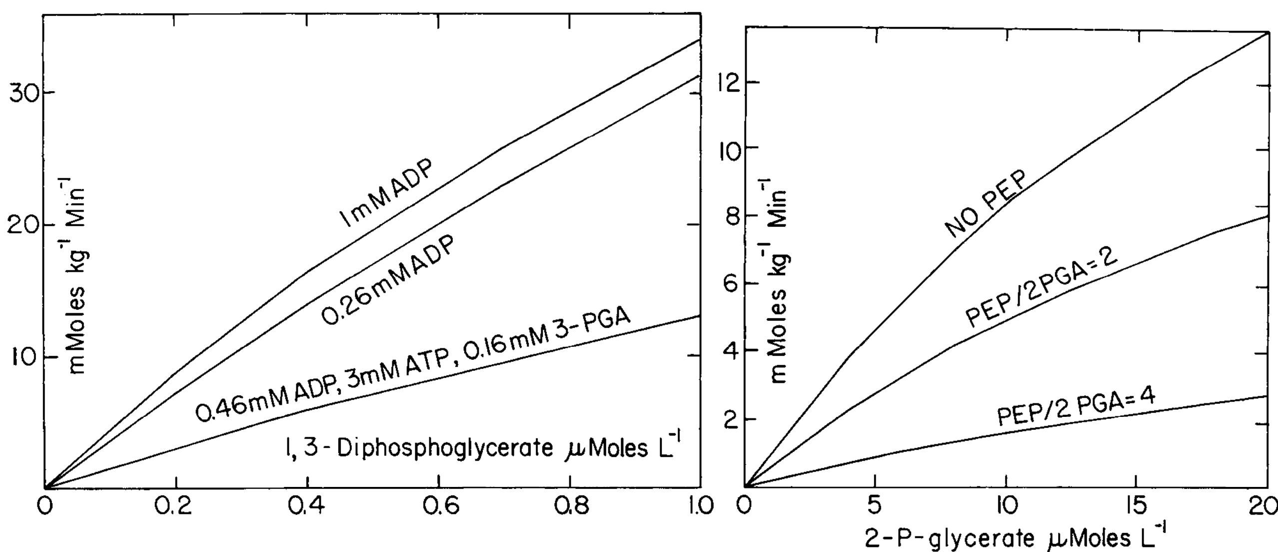
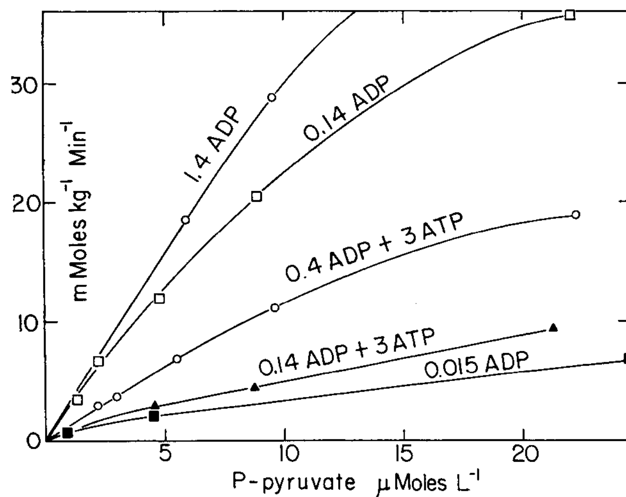



FIG. 4 (*left*). P-Glycerate kinase activity as a function of substrate concentration in the presence and absence of products of the reaction. These are theoretical curves calculated for 10-day-old mouse brain. For adults, multiply by 1.52. Calculations were based on kinetic constants determined at 30°, but which are believed to be nearly identical at 38° because of the very low temperature coefficient when substrate is low (Table III). *3-PGA*, 3-phosphoglycerate.

FIG. 5 (*right*). Theoretical velocities of enolase in 10-day-old mouse brain as a function of 2-P-glycerate (*2PGA*) concentration. For adults, multiply by 1.72. The effect of P-enolpyruvate (*PEP*) on the rate has been calculated on the assumption that the back-reaction is equal to the forward reaction multiplied by (P-enolpyruvate)/5(2-P-glycerate), where 5 is the equilibrium constant. This assumption is valid when substrate levels are low compared to Michaelis constants. This is the case for 2-P-glycerate, and is probably also the case for P-enolpyruvate, although the $K_s$ in the reverse direction was not measured.



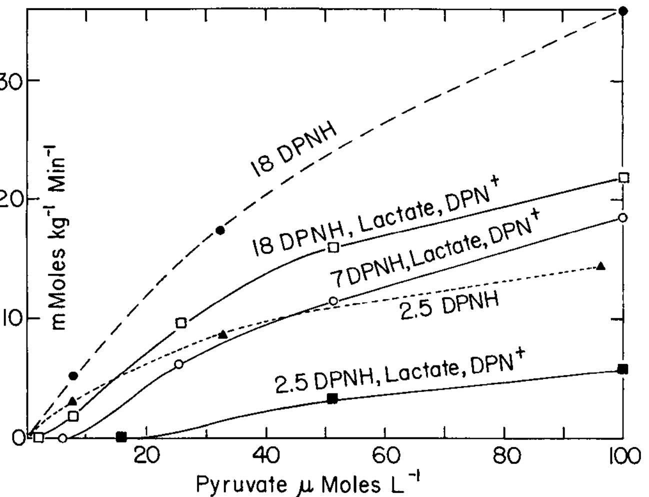




Fig. 6 (*left*). Pyruvate kinase activity as a function of P-enolpyruvate, ADP, and ATP concentrations. The velocities are calculated on the basis of what they would be in 10-day-old mouse brain from measurements *in vitro* at each of the P-pyruvate concentrations indicated by *points* on the *curves*. For adults, multiply by 1.75.

Fig. 7 (*right*). Lactic dehydrogenase activity in mouse brain. The activities are calculated to the enzyme content of 10-day-old brain from measurements *in vitro* at 28°, made at each of the pyruvate levels indicated by *points* on the *curves*. For adults, multiply by 1.09. DPNH concentrations are micromolar. Lactate and $DPN^+$, when added, were 3 and 0.3 mM, respectively. Intercepts with the *abscissa* correspond to equilibrium mixtures. No attempt has been made to correct to 38°. Because of increase in $K_s$ for pyruvate with temperature, values in this pyruvate concentration range of the figure may not be much higher at 38°.

for the present purpose than theoretical kinetic constants are the velocities with low P-pyruvate levels and various ADP and ATP concentrations (Fig. 6).

*Lactic Dehydrogenase*—As in the previous case, the $K_s$ values for each substrate were increased by increases in the other (Table III). In the cell, the products of pyruvate reduction are usually in great excess. Therefore, kinetic measurements were made in the presence of lactate and $DPN^+$ at about the concentrations found in brain (Fig. 7).

### *Equilibrium Constants*

The analyses were all carried out with the same enzymatic procedures used for the substrate measurements in brain (1).

*Fructose Diphosphate, Dihydroxyacetone-P, and Glyceraldehyde-P*—An equilibrium ratio for dihydroxyacetone-P to glyceraldehyde-P of 28:1 was observed. This is the average of three experiments (not shown), which gave values of 30, 31, and 24. Meyerhof and Junowicz-Kocholaty (27) found an average ratio of 24. The fructose diphosphate-triose-P equilibrium was established either with aldolase alone or with aldolase plus triose-P isomerase in order to bring the triose phosphates also into equilibrium with each other (Table IV). The reactions were stopped with HCl. In the longer incubations with high substrate levels, there was some destruction of triose-P and some sign of contamination of aldolase with triose-P isomerase. The best value from the data for the equilibrium constant is 90 μM at 38°. This may be compared with values of 119 and 133 reported, respectively, by Herbert *et al.* (28) and Meyerhof and Junowicz-Kocholaty (27).

*P-Enolpyruvate, 2-P-Glycerate, and 3-P-Glycerate* (*Table V*)—The equilibrium constant obtained for the enolase step, 4.6, agrees fairly closely with that obtained by Wold and Ballou (29) (5.28 at 34°, pH 7.5, in 0.4 M KCl with 8 mM $MgSO_4$). The equilibrium constant for the mutase step, however, is much lower than previously reported (23, 30). The difference is attributed to difficulties with earlier analytical methods in measuring 2-P-glycerate in the presence of much larger quantities of 3-P-glycerate or P-pyruvate. The equilibrium proportions for 3-P-glycerate to 2-P-glycerate to P-pyruvate, under conditions intended to simulate those *in vivo*, are calculated to be 10:1:4.6.

*Comparison of Calculated with Observed Substrate Levels*—From the information presented as to activities and kinetic parameters for enzymes of mouse brain, it should be possible to calculate

Table IV

*Equilibria between fructose diphosphate, dihydroxyacetone-P, and glyceraldehyde-P*

Fructose diphosphate at three different concentrations was incubated at 38° in the medium used for kinetic studies. The crystalline muscle enzyme concentrations were: aldolase, 5 μg per ml; triose-P isomerase, 0.01 μg per ml (present as a mixture with 0.1 μg of glycero-P dehydrogenase per ml). All concentrations and $K_{eq}$ are micromolar.

| Incubation | Aldolase | | | | Aldolase + triose-P isomerase | | | |
|---|---|---|---|---|---|---|---|---|
| | FDP[a] | DHAP | GAP | $K_{eq}$[b] | FDP | DHAP | GAP | $K_{eq}$ |
| *min* | | | | | | | | |
| 35 | 2750 | 541 | 430 | 85 | 2200 | 1980 | 70 | 63 |
| 300 | 2180 | 730 | 236 | 79 | 1460 | 2000 | 63 | 86 |
| 10 | 246 | 156 | 153 | 97 | 128 | 524 | 18 | 72 |
| 15 | 248 | 159 | 144 | 92 | 121 | 532 | 22 | 97 |
| 10 | | | | | 3.5 | 102 | 4.6 | 106[c] |
| 15 | 151 | 44 | 39 | 114 | 3.9 | 100 | 5.7 | 93[c] |

[a] FDP, fructose diphosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde phosphate.

[b] $K_{eq}$ = (DHAP) (GAP)/(FDP).

[c] Because the glyceraldehyde-P levels for these two samples were too low for accurate measurement, $K_{eq}$ was calculated by substituting dihydroxyacetone-P/28 for glyceraldehyde-P.

steady state substrate levels for any particular glycolytic flux, on the assumption that enzymes and substrates are evenly distributed. The glycolytic flux during ischemia was measured directly (1) from the rates of increase in lactate, in a series of brains exposed to different periods of ischemia. The values were confirmed by the rates of decrease of glucose and glycogen. It was also possible to calculate the glycolytic flux just before onset of ischemia from the amount of glucose oxidation that would be needed to provide the observed rate of ~P expenditure *during* ischemia. (The ~P expenditure was calculated from the rates of use of ATP, ADP, P-creatine, glucose, and glycogen (1).) The figure obtained is an upper limit, since the use of ~P is undoubtedly increased by the stimulus of decapitation. Since ischemia resulted in at least 4- to 7-fold increases in glycolytic flux and since data are available for groups of animals with quite different energy requirements, a 25-fold range of fluxes is presented for comparison with actual substrate levels.

The substrate levels that are recorded in Tables VI and VII are average concentrations for whole mouse brain calculated on a wet weight basis. On the basis of water content, they would be 25% higher, and local concentrations must have been higher yet. They would also vary between different cells and different parts of cells. No attempt will be made at present to take this distribution problem into account. It may be pointed out, however, that when substrate levels are far below Michaelis constants, uneven distribution of either enzyme or substrate alone will not alter the rate of substrate use, although uneven distribution of both enzyme and substrate will do so. Conversely, when substrate levels are well above Michaelis constants, uneven distribution neither of substrate nor of enzyme will alter rates of substrate use (unless, of course, substrate and enzyme are kept apart).

In Tables VI and VII two sets of calculated values are given in most cases. The first value is the expected concentration for even distribution of enzyme and substrate if there is no product inhibition or back-reaction, and the second is the expected concentration when products, at representative levels, are also present. This is intended to bring out the probable influence on a given step of succeeding steps downstream. Assumed cosubstrate concentrations are representative of observed levels (1).

*Glucose*—The upper limit for initial rates of glycolytic flux (Table VI) are equal to only about 3% of maximal hexokinase activity (Table II) in the unanesthetized animals and 1.5% in the anesthetized group. The average initial ATP and glucose concentrations are such as to give nearly maximal rates if there were no barrier between enzyme and substrates. There does not seem to be direct evidence that ATP is kept away from hexokinase, although this has been proposed as a control mechanism (31, 32). Glucose, however, may be segregated from hexokinase, since average initial glucose levels are only 15 to 20% of plasma levels in unanesthetized animals; *i.e.* there is a substantial permeability barrier. If glucose-6-P inhibition rather than impermeability accounts for hexokinase control, this product must be highly concentrated in the neighborhood of the enzyme. The highest initial glucose-6-P levels are only 0.25 mM. If this substance were confined to a quarter of the brain volume, it would inhibit hexokinase 90% as shown above, but not 97% or more as demanded for initial rate control. In diaphragm, skeletal muscle (33), and heart (34, 35), both glucose transport and glucose phosphorylation appear to be control mechanisms.

TABLE V

*Equilibria between 3-P-glycerate, 2-P-glycerate, and P-pyruvate*

Incubations were carried out for 10 to 60 minutes at 38° in the medium used for kinetic studies. The crystalline muscle enzymes used were either enolase, 2 μg per ml, or P-glycerate mutase, 10 μg per ml, or both. The mutase was supplemented with a crude preparation of 2,3-diphosphoglycerate from red blood cells. The reactions were stopped with acid, and analyses were made enzymatically (1). Concentrations are micromolar. $K_{eq}$ 1 refers to (2-P-glycerate)/(3-P-glycerate); $K_{eq}$ 2 refers to (P-pyruvate)/(2-P-glycerate).

| Starting material | Enzymes | 3-P-Glycerate | 2-P-Glycerate | P-Pyruvate | $K_{eq}$ | |
|---|---|---|---|---|---|---|
| | | | | | 1 | 2 |
| 2-P-Glycerate | Enolase | | 77 | 380 | | 4.9 |
| | Enolase | | 91 | 370 | | 4.1 |
| P-Pyruvate | Enolase | | 86 | 418 | | 4.9 |
| | Enolase | | 100 | 420 | | 4.2 |
| 3-P-Glycerate | Mutase | 339 | 34 | | 0.100 | |
| | Mutase | 370 | 40 | | 0.108 | |
| | Enolase + mutase | 271 | 29 | 146 | 0.107 | 5.0 |
| | Enolase + mutase | 276 | 32 | 138 | 0.117 | 4.3 |
| 2-P-Glycerate | Enolase + mutase | 309 | 33 | 161 | 0.107 | 5.0 |
| | Enolase + mutase | 332 | 33 | 139 | 0.099 | 4.2 |
| P-Pyruvate | Enolase + mutase | 340 | 33 | 175 | 0.097 | 5.3 |
| | Enolase + mutase | 332 | 29 | 117 | 0.087 | 4.0 |
| Average | | | | | 0.102 | 4.6 |

*Glucose-6-P*—Comparison of P-glucoisomerase kinetic constants and activity with the flux rates leads to the prediction that glucose-6-P and fructose-6-P should not depart greatly from equilibrium (3:1 ratio). The ratios between these two compounds calculated from the equation given for Fig. 2 are, for initial times and times of peak glycolysis, respectively, 3.06 and 3.47 (adult), 3.06 and 3.29 (10-day-old), and 3.02 and 3.17 (anesthetized group). Observed ratios exceeded these values somewhat. This could mean that isomerase is not located at the point of glucose-6-P generation; however, the measurement of the low fructose-6-P levels during peak glycolysis is subject to some analytical error (1).

*Fructose-6-P*—The observed initial fructose-6-P levels are much greater than those required to give initial glycolytic rates

TABLE VI

*Observed substrate concentrations in mouse brain compared with those calculated from glycolytic fluxes, enzyme kinetics, and enzyme activities present*

The observed values and the hexose fluxes are from the preceding paper (1). The times represent the interval between decapitation and fixation by rapid freezing. For each group, the time of peak flux has been chosen. This peak flux is calculated from lactate levels in a series of brains frozen at different intervals after decapitation. Each initial flux is an upper limit calculated from the glucose oxidation required to support ~P expenditure (see the text and the preceding paper (1)). The calculations are based on the kinetic data given and the following assumed conditions.

*Glucose*—Calculation *a*, 3 mM ATP and no product; Calculation *b*, the same plus 0.5 mM glucose-6-P.

*Glucose-6-P*—Calculation *a*, no product; Calculation *b*, fructose-6-P to glucose-6-P ratio of 1:3.3 (*i.e.* 10% out of equilibrium).

*Fructose-6-P*—3 mM ATP, 5 mM $P_i$, 0.2 mM AMP.

*Fructose Diphosphate*—No product.

*Glyceraldehyde-P*—5 mM $P_i$, 0.3 mM $DPN^+$.

The case of glucose for the control 10-day-old mice may be chosen as an example of how the calculations were made. At 6 seconds post decapitation the hexose flux was 1.8 mmoles per kg per minute. From Fig. 1 it can be graphically determined that without product inhibition a glucose concentration of only 6 μM would be required to provide this hexokinase velocity. With an assumed concentration of 0.5 mM glucose-6-P present (in this case 12 times the observed concentration), inhibition would be about 75% (noncompetitive). The ordinate of Fig. 1 can be imagined to be multiplied by 0.25, and it would now be graphically estimated that 36 μM glucose would be required for the observed flux. The calculations for the adults are made similarly except that the ordinate of Fig. 1 is multiplied by 1.8 to adjust for higher adult hexokinase levels. Note that calculations have been made as if the brain were 100% water with even distribution of substrates and enzymes.

| Experimental group | Time after decapitation | Hexose flux observed | Substrate concentration[a] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Glucose (hexokinase) | | | Glucose-6-P (isomerase) | | | Fructose-6-P (P-fructokinase) | | Fructose diphosphate (aldolase) | | Glyceraldehyde-P (glyceraldehyde-P dehydrogenase) | |
| | | | Calculated | | Observed | Calculated | | Observed | Calculated | Observed | Calculated | Observed | Calculated | Observed |
| | | | *a* | *b* | | *a* | *b* | | | | | | | |
| | | *mmoles/kg/min* | μM | | | μM | | | μM | | μM | | μM | |
| Adult | Initial | 0.76 | 1.2 | 5.5 | 1540 | 0.8 | 8 | 80 | 6 | 16 | 1 | 120 | 0.3 | 1.6[b] |
| | 4 sec | 5.65 | 10 | 193 | 1270 | 5 | 150 | 66 | 52 | 11 | 11 | 216 | 2.0 | 1.9[b] |
| 10-Day-old | | | | | | | | | | | | | | |
| Control | Initial | 0.40 | 1.2 | 5.1 | 750 | 0.6 | 6 | 91 | 4 | 23 | 0.8 | 109 | 0.2 | 0.5[b] |
| | 6 sec | 1.8 | 6 | 36 | 770 | 2.5 | 38 | 40 | 20 | 12 | 4 | 170 | 0.9 | 1.4[b] |
| Anesthetized | Initial | 0.23 | 0.7 | 2.8 | 2560 | 0.4 | 3 | 224 | 2.2 | 50 | 0.5 | 27 | 0.1 | 0.9[c] |
| | 25 sec | 1.65 | 6 | 31 | 1930 | 2.3 | 34 | 91 | 18 | 27 | 3 | 153 | 0.8 | 3.3[c] |

[a] The observed levels are per kg of wet brain. Because of uncertainties regarding extracellular volumes and substrate compartmentation, no attempt is made to calculate true concentrations on a water basis.

[b] Calculated from observed dihydroxyacetone-P on the assumption that equilibrium existed.

[c] Observed values. The value of 3.3 is for 60 seconds rather than 25 seconds post decapitation.

if P-fructokinase were fully active. This is particularly true of the anesthetized 10-day-old mice (Table VI), for which it is more likely than for other groups that freezing was fast enough to capture initial values. The paradoxical decrease in fructose-6-P levels in each group, as the flux increases, shows that phosphorylation of this substrate has been facilitated in some manner. As mentioned above, the peculiar kinetic properties of P-fructokinase appear to offer an explanation for this phenomenon. In any event, this step is an obvious control point in glycolysis, as shown for other tissues by a number of authors (references are cited in the preceding paper (1)).

*Fructose Diphosphate*—There is a very great discrepancy at all times between observed fructose diphosphate levels and those necessary for the observed fluxes (Table VI). High levels of this substrate would be expected if there were a limiting step further down the line. In this case, the lower the glycolytic flux, the more nearly would fructose diphosphate and the triose phosphates approach equilibrium. Superficially this does not seem to be true. Calculated equilibrium concentrations of triose phosphates, corresponding to observed fructose diphosphate levels at initial and peak glycolysis times in the anesthetized group, would be, respectively, 260 and 620 μM for dihydroxyacetone-P and 9 and 22 μM for glyceraldehyde-P, *i.e.* 10 or 20 times higher than observed levels. The concentrations found for fructose diphosphate and dihydroxyacetone-P concur with those of Thorn *et al.* for rabbit brain (36). The discrepancies would be less if the substrates were confined to a small compartment of the cells (*cf.* Table IV), but this compartment could be no more than 1% of the brain volume if observed levels are equilibrium concentrations. Either the aldolase step does not proceed unimpeded or part of the fructose diphosphate is not in free solution. Evidence for the latter comes from the fact that even when glycolysis ceases 10 or 20 minutes after decapitation, owing to exhaustion of carbohydrate stores, the residual fructose diphosphate and triose phosphates were not in equilibrium (1). After 10 minutes, the substrate levels are so low as to make analyses difficult, and therefore mice were poisoned with iodoacetate and decapitated; after the heads were held for 30 minutes at 38° to exhaust all carbohydrate, the brains were homogenized and analyzed. With 0, 50, and 100 mg

iodoacetate per kg, the fructose diphosphate levels were 13, 96, and 248 $\mu$M, whereas dihydroxyacetone-P levels were only 21, 27, and 117 $\mu$M, rather than 180, 490, and 790 as predicted from the equilibrium constant. Hess and Chance (37) found, in ascites tumor cells poisoned with iodoacetate and given glucose, that fructose diphosphate and dihydroxyacetone-P levels approached equilibrium. In this case the absolute levels rose much higher than those reported here. Hess (5) concluded that the aldolase system is close to equilibrium in unpoisoned ascites tumor cells. However, this conclusion is based on analytical values for glyceraldehyde-P which actually exceed those for dihydroxyacetone-P. It is difficult to see how this could ever take place during active glycolysis (unless there is a bound form of glyceraldehyde-P). The simplest explanation for the present results in brain would be that a large portion of the fructose diphosphate is not free in solution.

*Dihydroxyacetone-P and Glyceraldehyde-P*—The glyceraldehyde-P levels were measured in only two instances and were found to be somewhat higher than predicted for equilibrium with dihydroxyacetone-P. The levels are so low that even a small amount of binding could distort the ratio. The binder could be glyceraldehyde-P dehydrogenase itself. In addition, glyceraldehyde-P readily combines with a variety of amines. During rapid glycolysis, free glyceraldehyde-P levels calculated from dihydroxyacetone-P levels are not much greater than those required by the fluxes and the kinetic constants of glyceraldehyde-P dehydrogenase (Table VI); *i.e.* this step may be far from equilibrium and therefore a primary determinant of the levels of fructose diphosphate and dihydroxyacetone-P as well as of glyceraldehyde-P. To provide the observed peak fluxes with the calculated glyceraldehyde-P levels, it would be necessary that neither DPN+ or $P_i$ be rate-limiting. That this is probably the case is in keeping with observed concentrations of these cosubstrates, which are well above the respective Michaelis constants.

*1,3-Diphosphoglycerate*—No data are available as to actual levels of this substrate, except that they are less than 1 $\mu$M. The concentrations must lie between those that would be in equilibrium with glyceraldehyde-P and those that would be in equilibrium with 3-P-glycerate. These may be calculated in the fashion of Bücher and Klingenberg (2) and of Hohorst, Reim, and Bartels (38), provided that an estimate of local DPNH concentration can be obtained. Bücher and coworkers have assumed that lactate and pyruvate are in equilibrium with DPN+ and DPNH. It will be argued below that this is unlikely in brain during peak glycolysis. It seems probable from kinetic data for both glycero-P dehydrogenase and lactic dehydrogenase that a DPNH concentration of about 2 $\mu$M is required. This would give a DPN+:DPNH ratio of 150. With an equilibrium constant for the glyceraldehyde-P dehydrogenase step of 0.6 liter mole$^{-1}$ at pH 7 (39) and the observed values for DPN+, $P_i$, and dihydroxyacetone-P, equilibrium values for 1,3-diphosphoglycerate of the order of 0.2 to 0.5 $\mu$M may be calculated. Equilibrium values for this substrate calculated from observed 3-P-glycerate, ATP, and ADP levels would be 0.03 and 0.09 $\mu$M in the case of the 10-day-old anesthetized mice during initial and peak glycolysis times, respectively. The kinetic properties of P-glycerate kinase are such as to make these low figures quite reasonable (Table VII). Hohorst *et al.* (38) observed an average value of 0.4 $\mu$M for 1,3-diphosphoglycerate in rat abdominal muscle.

*3-P-Glycerate*—The levels of this substrate are much higher than would be required if there were no product inhibition or back-reaction. This is in accord with the fact that observed ratios between 3- and 2-P-glycerate are close to the equilibrium figure (Table VII). Hohorst *et al.* found a ratio of 8:1 for the glycerates in rat muscle (4), but Hess found ratios of only 2:1 in ascites tumor cells, with or without added glucose (5).

*2-P-Glycerate*—To judge from the Michaelis constant and activity of enolase, the substrate levels are considerably higher than would be required if there were no back-reaction. Therefore it would be expected that here also equilibrium would be approached. The analytical figures for P-pyruvate do not support this. Ratios of 1:1 to 1.5:1 have been observed instead of nearly 5:1, as predicted from the equilibrium constant (see above). Hohorst *et al.* found a ratio of 3:1 in resting muscle, and this may have decreased during activity (a decrease with activity was observed in the P-pyruvate to 3-P-glycerate ratio (4)). Hess observed ratios of 1.5:1 in ascites tumor cells both with and without glucose (5).

TABLE VII

*Observed substrate concentrations in brains of 10-day-old anesthetized mice compared with those calculated from glycolytic fluxes, enzyme kinetics, and enzyme activities present*

These data and the data in the last two lines of Table VI are from the same mice. Calculations are based on the following assumptions.

*1,3-Diphosphoglycerate*—Calculation *a*, 0.5 mM ADP; Calculation *b*, the same plus 3 mM ATP and 160 $\mu$M 3-P-glycerate.

*3-P-Glycerate*—Calculation *a*, no product; Calculation *b*, substrate to product ratio of 10.5:1.

*2-P-Glycerate*—Calculation *a*, no product; Calculation *b*, substrate to product ratio of 1:4.

*P-Pyruvate*—Calculation *a*, no product; Calculation *b*, 3 mM ATP.

*Pyruvate*—Calculation *a*, 2.5 $\mu$M DPNH and no products; Calculation *b*, 2.5 $\mu$M DPNH plus 3 mM lactate and 0.3 mM DPN+.

*Dihydroxyacetone-P*—Calculation *a*, 2.3 mM DPNH and no product; Calculation *b*, 0.22 mM L-$\alpha$-glycero-P and 0.3 mM DPN+.

See Table VI for fuller explanation.

| Substrate | Time after decapitation | Substrate concentration | | |
|---|---|---|---|---|
| | | Calculated | | Observed |
| | | *a* | *b* | |
| | | $\mu$M | | |
| 1,3-Diphosphoglycerate | Initial | 0.006 | 0.015 | $<1$ |
| (3-P-glycerate kinase) | 25 sec | 0.04 | 0.10 | $<1$ |
| 3-P-Glycerate (mutase) | Initial | 0.4 | 9 | 25 |
| | 25 sec | 2.3 | 71 | 85 |
| 2-P-Glycerate (enolase) | Initial | 0.2 | 1.2 | 2.8 |
| | 25 sec | 1.6 | 10.0 | 8.8 |
| P-Pyruvate (pyruvate | Initial | 0.1 | 0.4 | 3.5 |
| kinase) | 25 sec | 0.6 | 2.3 | 8.5 |
| Pyruvate (lactic dehydrogenase | 25 sec | 7.1 | 35 | 72 |
| Dihydroxyacetone-P[a] | Control | 13 | 33 | 39 |
| ($\alpha$-glycero-P dehydrogenase) | Anesthetized | 12 | 33 | 39 |

[a] Substrate levels at the time of maximal glycero-P flux. This maximal flux was almost the same for control and anesthetized 10-day-old mice, 0.10 mmole per kg per minute. There is no basis for calculating glycero-P flux at zero time.

Although the data are believed to be accurate, the levels of 2-P-glycerate are so low that the possibility of some analytical difficulty, such as contamination of enolase with another enzyme activity, has to be considered.

*P-Pyruvate*—The concentrations of this substrate are much higher than would be necessary for the measured fluxes as calculated from kinetic constants for pyruvate kinase. Possibly the local ADP level may become limiting. On the other hand, even if tissue levels were as low as predicted on kinetic grounds, they would nevertheless be much higher than equilibrium values. Based on the value of Krimsky for the equilibrium constant (40) and the observed values for pyruvate, ADP, and ATP, the equilibrium values for P-pyruvate in the anesthetized group would be 0.016 and 0.026 $\mu$M at 0 and 25 seconds after decapitation instead of 3.5 and 8.5 $\mu$M as observed. Hess also found that analytical values for P-pyruvate in glycolyzing ascites tumor cells were not compatible with equilibrium at this step (5). The kinetic data make it clear that, unlike P-glycerate kinase, pyruvate kinase does not have the capacity to maintain equilibrium even under oxidative conditions (Table VII). During peak glycolysis, the P-pyruvate level needs to be 100 times above the calculated equilibrium concentration to support the observed flux.

*Pyruvate*—Consideration of observed substrate levels, kinetic constants, and the equilibrium constant makes it doubtful that pyruvate and lactate are maintained near equilibrium during peak glycolysis. At pH 7 and 38°, ($DPN^+$)(lactate)/(DPNH)-(pyruvate) is about 20,000 at equilibrium (41). Observed levels of $DPN^+$, lactate, and pyruvate for 10-day-old anesthetized mice after 25 seconds of ischemia would yield an equilibrium value of 0.4 $\mu$M for DPNH. This is too low by a factor of about 5 to provide the observed lactate flux (Table VII; Fig. 7). Presumably DPNH is therefore at least 5 times higher locally than the equilibrium figure would predict.

*Glycero-P Flux*—The increase in lactate induced by anoxia is accompanied by a smaller but almost proportional increase in glycero-P (1). The peak rates of glycero-P flux were about 0.10 mmole per kg per minute in both control and anesthetized 10-day-old mice, *i.e.* about 6% of the maximal lactate flux. This may be compared with the maximal velocity of glycero-P dehydrogenase, which is only 1.5% of that of lactic dehydrogenase (Table II), and with the tissue first order rate constant for glycero-P dehydrogenase (Table III), which is 5.6% of that for lactic dehydrogenase. The two enzymes presumably operate from the same DPNH pool against the same $DPN^+$ concentration. The factor that may favor glycero-P formation is the relatively low glycero-P concentration, only 0.04 that of lactate at the time of peak flux for both substrates (1). As in the case of lactic dehydrogenase, kinetic considerations would seem to rule out a close approach to equilibrium during peak glycero-P flux. At 37° and pH 7, the equilibrium constant for (glycero-P)-($DPN^+$)/(dihydroxyacetone-P)(DPNH) is about 11,000 (41). If DPNH is in equilibrium with glycero-P, dihydroxyacetone-P, and $DPN^+$, it is present during peak glycolysis (10-day-old animals) at about 0.05 $\mu$M. This is 50 times less than the level required for the observed glycero-P flux (Table VII; Fig. 8).

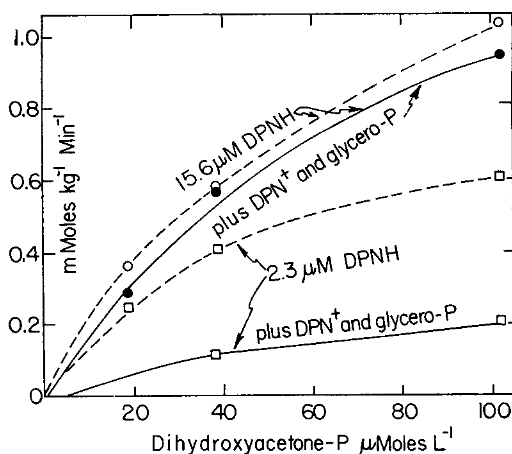



FIG. 8. Glycero-P dehydrogenase activity as a function of dihydroxyacetone-P and DPNH concentrations. $DPN^+$ and DL-$\alpha$-glycero-P were added as indicated at concentrations of 300 and 450 $\mu$M, respectively. The velocities are calculated for average 10-day-old mouse brain from measurements made at high dilution of an adult brain. For adults, multiply by 2.0.

## DISCUSSION

From data on substrate levels (1), it was concluded that the only true control points between glucose and pyruvate in brain are at the hexokinase and P-fructokinase steps. The degree to which kinetic information may explain this control has been discussed above.

The kinetic constants and substrate level measurements combined make it probable that even with maximal glycolytic flux, equilibrium is approximated at the hexose isomerase and P-glycerate mutase step, and kinetic considerations, at least, argue for near equilibrium at the aldolase and enolase steps. The glyceraldehyde-P dehydrogenase and P-glycerate kinase steps cannot be discussed with as much assurance. It is clear that one or the other or both must depart from equilibrium. The evidence seems to favor equilibrium for the kinase step, whereas the dehydrogenase step, at least during rapid glycolysis, may be far from equilibrium and an important determinant of fructose diphosphate and dihydroxyacetone-P concentrations.

The conclusions about these six enzymes agree with those of Hohorst *et al.* for stimulated muscle (4) and of Hess for ascites tumor cells (5), except that Hohorst *et al.* believed that glyceraldehyde-P dehydrogenase was never far from equilibrium and conversely Hess decided that P-glycerate kinase was far from equilibrium. The latter found a level for 1,3-diphosphoglycerate of 80 $\mu$M during active glycolysis. This is at least 80 times higher than the level in brain, and 200 times the level found by Hohorst *et al.* for resting muscle (38).

Kinetic reasons have been given for believing that there is lack of equilibrium of the $\alpha$-glycero-P and lactic dehydrogenase steps during peak glycolysis. This differs from the conclusions of Bücher *et al.* (2–4) concerning a number of other biological systems. These investigators found that initially and during sudden increase in glycolysis the ratios (lactate)/(pyruvate), (glycero-P)/(dihydroxyacetone-P), and (malate)/(oxaloacetate) are related to each other in the manner expected if all three systems were in equilibrium with a common pool of $DPN^+$ and DPNH. (This was not found true for the lactate and glycero-P systems in the present study.) It is possible that during rapid glycolysis, even if equilibrium were not attained, it might appear to be reached if these dehydrogenases, which utilize DPNH from the same pool, are present at levels proportionate to biological requirements.

As reported for ascites tumor cells (5), the pyruvate kinase step in brain is definitely not at equilibrium in terms of observed total tissue substrate levels, nor would the kinetic constants make equilibrium seem likely. Therefore pyruvate kinase may be a major factor in maintaining 2-P-glycerate, 3-P-glycerate, and perhaps 1,3-diphosphoglycerate at higher levels than would be the case if there were nothing to back up the stream.

Kinetic properties and amounts of enzymes are presumably determined by selective evolutionary processes. It is interesting to see that kinetic constants of the enzymes concerned match the thermodynamic needs of the system. For example, under almost any reasonable conditions, because of the equilibrium constants, 3-P-glycerate, 2-P-glycerate, glyceraldehyde-P, and 1,3-diphosphoglycerate would necessarily form a series with decreasing concentrations in the order given. Consequently the turnover rate for each substrate must *increase* in the same order. The measure of turnover capability is the tissue first order rate constant. It is found that for each of the four substrates above, the rate constants fall in exactly the required order (Table III).

The present study has been a comparison between kinetic parameters and average substrate levels for the whole brain. The brain is histologically the most heterogeneous organ in the body, and each of its cells has a complicated structure. Consequently, true local concentrations undoubtedly vary greatly and are quite different from average concentrations. Therefore, although it is believed that the broad outlines of the glycolytic stream in brain have been demonstrated, an understanding of the finer details must wait on histochemical studies of individual cells and parts of cells. It is in preparation for such studies that this gross work was undertaken.


### SUMMARY

1. All of the enzymes in mouse brain of the Embden-Meyerhof pathway except triosephosphate isomerase were studied. The maximal velocities at pH 7.1 were measured for both adult and 10-day-old mice in a medium intended to simulate ionic conditions *in vivo*. In the same medium, the kinetic constants were measured for each of the enzymes.

2. Equilibrium values for the aldolase, phosphoglycerate mutase, and enolase steps have been redetermined. The only discrepancy with earlier findings is that the equilibrium ratio of 3-phosphoglycerate to 2-phosphoglycerate appears to be 10:1 instead of 6:1 or less.

3. The results have been used to evaluate data from brains in which different rates of glycolysis were induced by ischemia or anesthesia. Comparisons have been made between observed concentrations of substrates and those calculated from the kinetic constants and the over-all glycolytic rates.

4. Hexokinase and phosphofructokinase occupy special control positions, the only ones demonstrated between glucose and pyruvate by the present experiments with brain.

5. The steps catalyzed by phosphoglucoisomerase and phosphoglycerate mutase are probably maintained near equilibrium even during maximal flux. On kinetic grounds, the same seems true for enolase and aldolase in spite of the fact that observed phosphopyruvate levels are too low and fructose diphosphate levels are much too high for equilibrium ratios. The possibility of bound or sequestered fructose diphosphate is suggested.

6. Kinetic evidence indicates that the reactions catalyzed by lactic dehydrogenase and glycerophosphate dehydrogenase are not maintained at equilibrium during peak glycolysis, and the pyruvate kinase step is almost certainly not at equilibrium either under oxidative or glycolytic conditions. It seems probable that this step maintains the tissue levels of 2-phosphoglycerate, 3-phosphoglycerate, and possibly 1,3-diphosphoglycerate at higher levels than would otherwise be the case. In a similar manner, glyceraldehyde phosphate dehydrogenase may be a factor in raising the tissue levels of fructose diphosphate and dihydroxyacetone phosphate.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