The Rate-Determining Nature of Aldolase in Glycolysis by Rat-Brain Preparations

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It has been shown that the addition of rat-brain mitochondria to an equivalent amount of rat-brain cell sap, supplemented by an optimum amount of added yeast hexokinase, will stimulate glycolysis; the addition of aldolase also stimulated the glycolytic rate (Brunngraber & Abood, 1960). The deduction that aldolase is a rate-limiting enzyme in these glycolytic systems has been challenged by Johnson (1962), who found that his glycolytic system was partially inactivated when incubated in a medium lacking sulphhydryl groups. On the basis of these results he deduced that the stimulation produced by aldolase or mitochondria was an artifact. The rat-brain glycolytic system has therefore been re-investigated by us in order to find an explanation for the different results obtained in the two Laboratories. The present paper presents evidence that inactivation is not observed in our glycolytic systems, nor does added glutathione stimulate glycolysis. Additional evidence is presented demonstrating that aldolase is indeed a rate-limiting enzyme. However, the aldolase is rate-limiting only when exogenous hexokinase supplements the medium. In the absence of added hexokinase, the primary rate-determination steps are the hexokinase reaction itself and the rate-determining effect occasioned by lack of the availability of ADP.

EXPERIMENTAL

Special chemicals and reagents. Crystalline yeast hexokinase, triose phosphate isomerase and 3-phosphoglycerate (barium salt) were obtained from C. F. Boehringer und Soehne, Mannheim, Germany. Crystalline aldolase and glyceraldehyde 3-phosphate dehydrogenase, ATP, ADP, NADH, fructose 6-phosphate (sodium salt), glucose 6-phosphate (sodium salt), fructose 1,6-diphosphate (sodium salt) and deoxyglucose were obtained from the Sigma Chemical Company, St Louis, Mo., U.S.A. 2,3-Diphosphoglycerate (barium salt) was obtained from Schwartz Laboratories Inc., U.S.A.

Preparation of enzymes. One whole rat brain was homogenized in 10 ml. of 0.25M-sucrose containing EDTA (sodium salt) (0.5 mM), pH 7.0, and centrifuged at 100 000g for 20 min. The protein content of the supernatant (cell sap) averaged 4.45 ± 0.22 mg. of protein/ml. (12 experiments). The 'mitochondrial' fraction was prepared from 10 ml. of homogenate as described by Brunngraber &

Abood (1960) and was suspended in 10 ml. of water. The protein concentration of the 'mitochondrial' suspension averaged 8.45 ± 1.35 mg. of protein/ml. (8 experiments). Equal volumes of cell sap and 'mitochondrial' suspension provided a system capable of accomplishing the same amount of glycolysis as an equivalent volume of the original homogenate (Abood, Brunngraber & Taylor, 1959).

Enzyme assays. All incubations were made in Warburg flasks in an atmosphere of $N_2 + CO_2$ (95:5). The glycolytic medium and enzymes (at 0°) were mixed and 3 ml. samples pipetted into Warburg flasks and placed in the Warburg bath. Flasks were removed at appropriate intervals, and the solutions were deproteinized with 0.1 ml. of 50% (w/v) trichloroacetic acid and analysed for lactate, pyruvate, fructose 1,6-diphosphate and triose phosphates. The standard glycolytic medium (medium A) contained: potassium phosphate buffer, pH 7.5 (8 mM); ATP (0.5 mM); magnesium chloride (7 mM); NAD (0.36 mM); potassium bicarbonate (17 mM); nicotinamide (27 mM); glucose (15 mM). When included in the medium the final concentration of fructose 1,6-diphosphate, glucose 6-phosphate and fructose 6-phosphate was 4.1 mM in each case.

The formation of pyruvate from 3-phosphoglycerate was followed in a similar fashion, except that the medium (medium B) consisted of: potassium phosphate buffer, pH 7.5 (8 mM); ADP (0.5 mM); potassium bicarbonate (17 mM); magnesium chloride (7 mM); 3-phosphoglycerate (13.3 mM). To regenerate ADP from ATP, deoxyglucose (20 mM) and hexokinase were included in the incubation medium.

To measure the rate of formation of fructose 1,6-diphosphate and of triose phosphates from glucose, glucose 6-phosphate and fructose 6-phosphate, incubation was performed in test tubes (open to the atmosphere) in a medium (medium C) containing: magnesium chloride (15 mM); potassium phosphate buffer, pH 7.4 (10 mM); ATP (10 mM). The substrates were fructose 6-phosphate (10 mM), glucose 6-phosphate (10 mM) or glucose (30 mM). The reaction was terminated by the addition of 0.1 ml. of 50% (w/v) trichloroacetic acid, and the supernatant obtained after centrifuging was analysed for fructose 1,6-diphosphate and triose phosphates.

The final volume of all incubation media, after the addition of enzymes, was 3 ml. The amount of cell sap included in the medium was generally 0.1, 0.2 or 0.3 ml.

When aldolase or hexokinase was added to the incubation medium, care was taken to maintain a constant ratio of added enzyme to the volume of rat-brain cell sap used in the assay. These ratios were: 20 μ g. of crystalline yeast hexokinase/ml. of the original rat-brain cell sap, and 335 μ g. of aldolase/ml. of the original rat-brain cell sap.

Table 1. *Effect of added aldolase and rat-brain mitochondria on the rate of glycolysis of rat-brain cell sap supplemented by yeast hexokinase*

Various amounts of cell sap supplemented with optimum amounts of hexokinase, either alone or with added aldolase or mitochondria, were incubated in the presence of the standard glycolytic medium (medium A; see the Experimental section), containing fructose 1,6-diphosphate and glucose, for various time-intervals. The activity was obtained from the slope of the curve of the formation of lactate plus pyruvate plotted against time (five points). This curve is linear after an initial lag period.

Expt. no.	Enzyme system	Vol. of cell sap added (ml./flask)	Rate of formation of lactate + pyruvate (μ mole/flask/min.)
1	Cell sap + hexokinase	0.1	0.152
		0.2	0.300
		0.3	0.466
	Cell sap + hexokinase + mitochondria	0.1	0.250
		0.2	0.500
2	Cell sap + hexokinase	0.1	0.113
		0.2	0.257
		0.3	0.388
	Cell sap + hexokinase + aldolase	0.1	0.175
		0.2	0.343

Excess, as well as a deficiency, of added enzyme will produce glycolytic rates lower than maximal. When mitochondria were added to the incubation medium, a volume equal to that of the cell sap was used. Thus 1 ml. of cell sap plus 1 ml. of mitochondria in the incubation medium was equivalent to 1 ml. of homogenate, with the nuclei, microsomes, red blood cells etc. excluded.

Analytical methods. Lactate was determined by the method of Barker & Summerson (1941). Pyruvate was determined by the method of Tonhazy, White & Umbreit (1950). Fructose 1,6-diphosphate was determined by the method described by Aisenberg (1959). Triose phosphates were determined by a method described by Racker (1957). ATP was determined by an enzymic method, based on the unpublished work of Bücher & Schuartz, with kit TC-J 15979 purchased from C. F. Boehringer und Soehne, Mannheim, Germany; this method depends on the assay of NAD^+ formed in the sequence of reactions catalysed by phosphoglycerate kinase and 3-phosphoglycerdehyde dehydrogenase in the presence of NADH, ATP and excess of 3-phosphoglycerate.

RESULTS

Effect of the addition of hexokinase and aldolase to rat-brain cell sap on the rate of production of lactate. A ratio of 20 μ g. of yeast hexokinase to 1 ml. (4.45 mg. of protein) of the original rat-brain cell sap/Warburg flask incubated in the presence of glucose as sole substrate in medium A provided maximal accumulation of lactate plus pyruvate. Concentrations of added hexokinase much above or below this ratio gave lower rates of accumulation of lactate. Rat-brain cell sap in the presence of an added optimum amount of hexokinase with fructose 1,6-diphosphate and glucose as substrates in medium A gave an additional increase in the rate of formation of lactate when aldolase was added.

A maximal rate of accumulation of lactate was obtained when 335 μ g. of aldolase/ml. of the original cell sap was used in the assay. Concentrations of added aldolase much above or below this ratio gave lower rates of accumulation of lactate. These results confirmed those obtained by Brunngraber & Aboud (1960).

Effect of the addition of aldolase or an equivalent amount of rat-brain mitochondria on glycolysis by the cell sap. Table 1 summarizes the effect of added aldolase or mitochondria on the rate of accumulation of lactate plus pyruvate by rat-brain cell sap supplemented with an optimum amount of yeast hexokinase. The activity is linear with enzyme concentration. A report (Johnson, 1962) of inactivation of brain glycolytic enzymes at dilute enzyme concentrations was not confirmed. The results of Table 1 indicate a 65% stimulation by an equivalent amount of 'mitochondrial' suspension and 33–55% stimulation by aldolase.

In the cell sap-plus-hexokinase system, the maximal accumulation of triose phosphates in the incubation medium was 0.40 μ mole/Warburg flask. When aldolase or mitochondria were added, the accumulation increased to 2.25 μ moles and 0.90 μ mole/Warburg flask respectively. This amount was reached in 15–30 min. and remained constant for the remainder of the incubation. If aldolase is rate-limiting, one might expect a low concentration of triose phosphates, which are being removed as rapidly as they are formed. The addition of aldolase eliminates this rate-limiting step, and the concentration of triose phosphates will rise since the rate-limiting reaction will now lie at some point below the level of the triose phosphates in the glycolytic sequence.

Table 2 is a summary of the results obtained in the same kind of experiment but with glucose serving as the sole substrate. Fructose 1,6-diphosphate accumulated at a high rate in the cell sap-plus-hexokinase system, whereas the rate of accumulation of triose phosphates was low. The addition of aldolase decreased the rate of accumulation of fructose 1,6-diphosphate by 80–90 % but increased the rate of accumulation of triose phosphates by 150–350 %. This is to be expected if aldolase is the rate-limiting enzyme. The addition of mitochondria to the cell sap-plus-hexokinase system appeared to have no effect on the rate of accumulation of fructose 1,6-diphosphate, although the rate of formation of lactate plus pyruvate was stimulated by 82 %. It appeared that aldolase is also rate-limiting when the cell sap-plus-hexokinase system was supplemented by the addition of an equivalent amount of mitochondria. This is in agreement with the results obtained by Brunngraber & Abood (1960). The evidence that fructose 1,6-diphosphate accumulated in these glycolytic systems indicates that the objection by Johnson (1960) with regard to the inadequacy of the fructose 1,6-diphosphate concentration in the media used in these experiments by Brunngraber & Abood (1960) is without foundation.

With the exception of the glycolytic system represented by the cell sap supplemented with hexokinase and mitochondria, no inactivation at low enzyme concentration was observed, and the stimulation by mitochondria was 82 % whereas that by aldolase was 14–24 %. Inactivation at low enzyme concentrations in systems in which glucose served as sole substrate might be explained by inactivation of phosphofructokinase, which is known to be unstable (Johnson, 1960). This would

not explain the inactivation observed in Johnson's (1962) glycolytic systems, however, since his media included fructose 1,6-diphosphate.

Effect of glutathione and ammonium sulphate on glycolysis. The addition of glutathione in concentrations used by Johnson (1962) to reactivate his glycolytic system resulted in approximately a 10 % inhibition of the formation of lactate plus pyruvate when fructose 1,6-diphosphate was used as substrate in the present work. Since the aldolase used in these experiments was a preparation suspended in ammonium sulphate, the effect of this salt on the glycolytic systems used was tested. Concentrations of ammonium sulphate included in the incubation medium comparable with that added with the aldolase had no effect. Concentrations greater than 5 mM inhibited glycolysis.

Mitochondrial stimulation of glycolysis by the cell sap. Various concentrations of cell sap, of mitochondria and of cell sap plus mitochondria (all supplemented with an optimum amount of yeast hexokinase) were incubated for 45 min. and the rate of accumulation of lactate plus pyruvate was determined. Fructose 1,6-diphosphate and glucose served as substrates. The activity, determined from the linear portion of the curve and expressed as μ moles of lactate plus pyruvate formed/Warburg flask/ml. of enzyme/min., was 0.83, 0.13 and 1.17 for cell sap, mitochondria and cell sap plus mitochondria respectively. Mitochondrial stimulation (percentage increase in glycolysis of the cell sap-plus-mitochondria system compared with the cell sap alone) was 41 % in this experiment. According to Johnson's (1960) definition of stimulation (the percentage increase in glycolysis of the cell sap-plus-mitochondria system compared with the sum of the glycolytic activities of the mito-

Table 2. *Rate of formation of lactate plus pyruvate from glucose by rat-brain cell sap supplemented with added yeast hexokinase and the effect of added aldolase or rat-brain mitochondria*

Various concentrations of cell sap supplemented with optimum amounts of hexokinase and added aldolase or mitochondria were incubated in the presence of the standard glycolytic medium (medium A; see the Experimental section), containing glucose as the sole substrate, for various time-intervals. The activity was obtained from the slope of the curve of the rate of formation of product (pyruvate plus lactate, triose phosphates or fructose 1,6-diphosphate) plotted against time (five points). The curve is linear after an initial lag period.

Enzyme system	Vol. of cell sap (ml.)	Rate of formation (μ mole/flask/min.)		
		Lactate + pyruvate	Triose phosphate	Fructose 1,6-diphosphate
Cell sap + hexokinase	0.1	0.097	0	0.009
	0.2	0.180	0.002	0.02
	0.3	0.283	0.002	0.03
Cell sap + hexokinase + aldolase	0.1	0.105	0.002	0
	0.2	0.222	0.005	0.002
	0.3	0.320	0.009	0.006
Cell sap + hexokinase + mitochondria	0.1	0.108	0	0.002
	0.2	0.327	0.003	0.017
	0.3	0.510	0.006	0.03

Table 3. *Rates of accumulation of lactate plus pyruvate, of fructose 1,6-diphosphate and of triose phosphate from glucose by rat-brain cell sap incubated alone and the effect of supplementation with yeast hexokinase and rat-brain mitochondria*

The rates represent the slope of formation of product plotted against time. These rates were linear after an initial lag period. Glucose was the sole substrate in the medium (medium A, see the Experimental section).

Enzyme system	Rate of accumulation (μ moles/flask/ml. of cell sap/min.)			ATP:ADP + AMP ratio
	Lactate + pyruvate	Fructose 1,6-diphosphate	Triose phosphate	
Cell sap	0.166	0	0	21
Cell sap + hexokinase	0.771	0.078	0.01	0.56
Cell sap + mitochondria	0.625	0	0.0081	4.1
Cell sap + hexokinase + mitochondria	1.17	0.084	0.015	1.2

chondria and cell sap alone), mitochondrial stimulation is calculated to be 22%.

Effect of the addition of hexokinase to cell sap and cell sap-plus-mitochondria systems on glycolysis. The experiments reported above were conducted in the presence of optimum concentrations of added hexokinase. Hexokinase serves two functions in the systems described. First, it converts glucose into glucose 6-phosphate in the initial step in the glycolytic reaction sequence. Secondly, it provides a source of ADP required for reactions subsequent to the formation of fructose 1,6-diphosphate in the reaction sequence. The addition of an optimum amount of hexokinase to cell sap incubated with glucose as the sole substrate increased the rate of formation of lactate plus pyruvate approximately fivefold (Table 3), and the ATP:ADP-plus-AMP ratio decreased from 21 to 0.56. No accumulation of fructose 1,6-diphosphate occurred in the absence of added hexokinase; the rate-limiting character of aldolase was apparent only when the medium had been supplemented with hexokinase. The glycolytic activity of the cell sap-plus-mitochondria system was doubled by the addition of optimum amounts of hexokinase, and the ATP:ADP-plus-AMP ratio was lowered from 4.1 to 1.2. No fructose 1,6-diphosphate accumulated in the absence of hexokinase, indicating again that the aldolase did not appear to be rate-limiting in the absence of added hexokinase.

If the chief function of the added hexokinase is to provide a source of ADP in these systems, one might expect an accumulation of fructose 1,6-diphosphate in the absence of added hexokinase. It appears that the formation of glucose 6-phosphate is the rate-limiting step in the overall sequence, the amount of ADP available being sufficient for the utilization of the limited amount of hexose phosphate formed without an accumulation of fructose 1,6-diphosphate. The addition of hexokinase serves to eliminate this rate-limiting step, but it also lowers the ATP:ADP-plus-AMP

ratio, and aldolase becomes the rate-limiting enzyme in the overall sequence. In this experiment, the further addition of aldolase decreased the rate of accumulation of fructose 1,6-diphosphate from 0.078 to 0.011 μ mole/Warburg flask/ml. of cell sap/min. in the cell sap-plus-hexokinase system, and from 0.084 to 0.02 μ mole/Warburg flask/ml. of cell sap/min. in the same system supplemented with brain mitochondria.

Especially surprising is the high ATP:ADP-plus-AMP ratio of the cell sap-plus-mitochondria system, and the fact that the addition of mitochondria to the cell sap-plus-hexokinase system increased the ratio from 0.56 to 1.2. The mitochondria contain the bulk of the hexokinase and adenosine-triphosphatase activities of the brain tissue, all of which might be expected to decrease the ratio. Apparently the ATP-forming enzymes present in the mitochondria more than compensated for these ATP-utilizers.

Table 4 provides additional findings giving evidence of the rate-limiting nature of brain hexokinase. For the cell sap alone (Expt. 2 of Table 4), in the absence of added hexokinase, the inclusion of glucose 6-phosphate or fructose 6-phosphate in the incubation medium tripled the rate of glycolysis and permitted an accumulation of fructose 1,6-diphosphate. Presumably the rate-limiting reaction in this case was the lack of available ADP. The fact that the rate of formation of lactate was increased confirms the analysis above that sufficient ADP is available to permit all of the hexose phosphate formed from glucose to be converted into lactate without an accumulation of fructose 1,6-diphosphate. When exogenous glucose 6-phosphate was added, however, the rate of glycolysis was increased to a point where ADP availability became the rate-limiting step. (More ADP was also being made available by the phosphofructokinase reaction.) Under these circumstances fructose 1,6-diphosphate and triose phosphates will accumulate. The fructose 1,6-diphos-

phate:triose phosphate ratio was low (1.3–1.9), as one would expect if ADP were the rate-limiting factor. The addition of hexokinase then lowered the ATP:ADP-plus-AMP ratio, increased the rate of glycolysis; aldolase became rate-limiting and fructose 1,6-diphosphate and triose phosphates accumulated. This time, as expected, the fructose 1,6-diphosphate:triose phosphates ratio was high (7.5); triose phosphates were now being removed as fast as aldolase permitted their formation.

The cell sap-plus-mitochondria system differs, however, since the addition of glucose 6-phosphate or fructose 6-phosphate did not increase the rate of glycolysis (Expt. 1 of Table 4). The low fructose 1,6-diphosphate:triose phosphates ratio (1.5–1.7) indicates that the rate-limiting step is a limitation of ADP. The equilibrium of the aldolase reaction lies at a point where about 10% of the fructose 1,6-diphosphate has been converted into triose phosphates. Similarly, the equilibrium point for the triose phosphate-isomerase reaction lies at a point where 95% of the triose phosphates is in the form of dihydroxyacetone phosphate. Calculation shows that the overall equilibrium for the two reactions together provides a mixture in which the fructose 1,6-diphosphate:triose phosphates ratio is 1.86. The ATP:ADP-plus-AMP ratio was 4.1 in this system. It is surprising therefore that fructose

1,6-diphosphate did not accumulate when glucose was the sole substrate. It appears that the situation is similar to that of the cell sap alone: the limited amount of glucose 6-phosphate formed is quickly utilized to form lactate without accumulation of fructose 1,6-diphosphate, since sufficient ADP is available. Since the addition of glucose 6-phosphate or fructose 6-phosphate did not increase the glycolytic rate in the cell sap-plus-mitochondria system (but did increase the rates of accumulation of fructose 1,6-diphosphate and of triose phosphates), it appears that the amount of ADP provided in this system is just sufficient to handle the amount of glucose 6-phosphate made available by the brain hexokinase. Another factor is the inhibition of brain hexokinase by glucose 6-phosphate. The addition of the latter compound to incubation media will therefore inhibit the formation of ADP by the hexokinase reaction. The decrease in glycolysis occasioned by this decreased availability of ADP will, to some extent, offset the increase in glycolysis expected by the addition of glucose 6-phosphate to the medium.

The addition of hexokinase to the cell sap-plus-mitochondria glycolytic system lowered the ATP:ADP-plus-AMP ratio, and again aldolase became rate-limiting as the rate of glycolysis was increased twofold. The fructose 1,6-diphosphate:

Table 4. *Rate of formation of lactate plus pyruvate, and the accumulation of fructose 1,6-diphosphate and of triose phosphates, at two time-intervals, from glucose, glucose 6-phosphate and fructose 6-phosphate by rat-brain cell sap and by cell sap supplemented with hexokinase and mitochondria*

The rate of formation of lactate plus pyruvate represents the slope of lactate plus pyruvate formed/Warburg flask plotted against time. These curves are linear after an initial lag period. The amounts of fructose 1,6-diphosphate and triose phosphates accumulated at two time-intervals are given. The composition of the medium (medium A) is given in the Experimental section.

Expt. no.	Enzyme system	Substrate	Rate of formation of lactate + pyruvate (μ mole/ml. of cell sap/min.)	Time (min.)	Fructose 1,6-diphosphate accumulated (μ moles/flask)	Triose phosphate accumulated (μ moles/flask)	Fructose 1,6-diphosphate: triose phosphate ratio
1	Cell sap + mitochondria	Glucose	0.48	30	0.15	0	—
				60	0.21	0.075	2.6
	Cell sap + mitochondria	Glucose and glucose 6-phosphate	0.41	30	2.06	0.81	2.4
				60	2.06	1.32	1.5
	Cell sap + mitochondria	Glucose and fructose 6-phosphate	0.43	30	1.94	0.80	2.4
				60	1.63	0.99	1.7
	Cell sap + mitochondria + hexokinase	Glucose	0.80	30	0.75*	0.21	3.6
				60	2.02*	0.38	5.3
2	Cell sap	Glucose	0.07	30	0	0	—
				60	0	0	—
	Cell sap	Glucose and glucose 6-phosphate	0.24	30	1.27	0.59	2.2
				60	2.09	1.11	1.9
	Cell sap	Glucose and fructose 6-phosphate	0.24	30	1.23	0.60	2.0
				60	2.05	1.58	1.3
	Cell sap + hexokinase	Glucose	0.55	30	0.34*	0.15	2.3
				60	1.05*	0.14	7.5

* In these incubations fructose 1,6-diphosphate and triose phosphate accumulated linearly with time, after an initial lag period, throughout the 60 min. incubation period. In the other experiments fructose 1,6-diphosphate and triose phosphates reached maximum concentration by 30–45 min., after which no further accumulation occurred.

triose phosphates ratio was high (5.3), as one would expect if aldolase were rate-limiting.

Formation of pyruvate from 3-phosphoglycerate. When mitochondria were incubated in the presence of 3-phosphoglycerate and ADP, pyruvate was formed (Table 5). The addition of cell sap caused only a slight increase in the rate of formation of pyruvate because the availability of ADP was rate-limiting. When hexokinase and deoxyglucose were added to provide an ADP-regenerating system, the rate of production of pyruvate in the mitochondrial system was increased only slightly, but the activity of the cell sap-plus-mitochondria system was increased approximately fourfold. Two observations are of interest. First, 75 % of the activity appears to reside in the cell sap and 25 % in the mitochondria. These results do not agree with those of Johnson (1960), who found 93 % of the 3-phosphoglycerate mutase and enolase were in the cell sap and only 7 % in the mitochondrial fraction. The activities he reported for these enzymes also appeared to be low. The activities reported in Table 5 exceed the highest glycolytic activities obtained when the formation of lactate from glucose or fructose 1,6-diphosphate was measured (Tables 1-4). It is concluded that the enzymes metabolizing 3-phosphoglycerate, 2-phosphoglycerate and phosphoenolpyruvate are not rate-limiting enzymes in the glycolytic scheme. The presence or absence of added 2,3-diphosphoglycerate appeared to make no difference in these experiments.

Formation of fructose 1,6-diphosphate from glucose. This reaction was studied under different conditions

and comparisons with results obtained from previous experiments must be made with caution. The incubations were carried out in test tubes (aerobically), with a high concentration of ATP. The formation of fructose 1,6-diphosphate and of triose phosphates from glucose, glucose 6-phosphate and fructose 6-phosphate at four different enzyme concentrations was measured after incubation for 30 min. The activity-enzyme concentration relation was linear when fructose 6-phosphate and glucose 6-phosphate served as the sole substrates; however, a 'lag period' indicating enzyme 'inactivation' occurred when glucose was the sole substrate. The value in Table 6 is an estimate of the activity based on only two points, and may be in error. Glucose 6-phosphate isomerase was not rate-limiting in relation to the phosphofructokinase. The activities of both of these enzymes exceeded the highest glycolytic activity observed in the usual glycolytic experiments when the formation of lactate from glucose was being measured. The hexokinase preparation used in these experiments did not contain these enzymes. The fact that phosphofructokinase and glucose 6-phosphate isomerase were not rate-limiting in the glycolytic scheme was demonstrated above (Table 2). The fructose 1,6-diphosphate:triose phosphates ratios were in the range 1.50-2.00, which is close to the expected equilibrium value of 1.86.

In all of the experiments reported in which hexokinase was added to brain glycolysis systems, the amount of hexokinase added was an 'optimum amount' that gave maximal glycolytic rates with cell sap and glucose as the sole substrate. Table 6 indicates that this amount of hexokinase was not present in excess. Phosphofructokinase and glucose 6-phosphate isomerase were twice as active as the added yeast hexokinase together with the endogenous hexokinase present in the cell sap. This supports the concept that the glucose 6-phosphate isomerase and phosphofructokinase were not rate-limiting enzymes in the overall sequence of reactions. The results of Table 6 indicate that one-third of the phosphofructokinase and glucose 6-phosphate isomerase of the brain homogenate is localized in the 'mitochondrial fraction'.

DISCUSSION

The present work confirms the findings by Brunngraber & Aboud (1960) that aldolase is rate-limiting in glycolysis by rat-brain cell sap and by the cell sap supplemented with an equivalent amount of rat-brain mitochondria when these systems are supplemented with added hexokinase. In the experiments described in the present work, mitochondrial stimulation of glycolysis by the cell sap is greater than the stimulation observed with added

Table 5. *Rate of formation of pyruvate from 3-phosphoglycerate by rat-brain mitochondria and cell sap*

The formation of pyruvate from 3-phosphoglycerate by rat-brain cell sap and mitochondria and the effect of added yeast hexokinase and deoxyglucose as an ADP-regenerating system were determined in medium B (see the Experimental section). The values were obtained from the curve of pyruvate formation plotted against enzyme concentration. These curves were linear and intersected the origin at zero enzyme concentration.

Expt. no.	Enzyme system	Rate of formation of pyruvate (μ moles/flask/ml. of cell sap or mitochondria/min.)
1	Mitochondria	0.90
	Mitochondria + cell sap	0.96
2	Cell sap + hexokinase	2.64
	Cell sap + hexokinase + mitochondria	3.54
	Mitochondria	0.90
	Mitochondria + hexokinase	1.05
3	Cell sap + hexokinase	2.58
	Mitochondria + hexokinase	0.75
	Cell sap + mitochondria + hexokinase	3.42

Table 6. *Rate of formation of fructose 1,6-diphosphate from glucose, glucose 6-phosphate and fructose 6-phosphate by rat-brain cell sap, mitochondria and cell sap plus mitochondria*

The formation of fructose 1,6-diphosphate and triose phosphates by rat-brain cell sap, mitochondria and cell sap plus mitochondria were obtained from the curve of the accumulation of fructose 1,6-diphosphate plus triose phosphates in 30 min. plotted against enzyme concentration. These curves were linear with time, with the exception noted. The composition of the medium (medium C) is given in the Experimental section. Two molecules of triose were counted as one molecule of fructose 1,6-diphosphate.

Expt. no.	Enzyme system	Substrate	Rate of formation of fructose 1,6-diphosphate (μ moles/flask/ml. of cell sap or mitochondria/min.)
1	Cell sap	Fructose 6-phosphate	1.92
2	Cell sap	Glucose 6-phosphate	1.96
3	Cell sap	Glucose	0.68*
4	Cell sap + hexokinase	Fructose 6-phosphate	1.61
5	Cell sap + hexokinase	Glucose 6-phosphate	1.78
6	Cell sap + hexokinase	Glucose	0.88
7	Mitochondria	Fructose 6-phosphate	0.94
8	Mitochondria	Glucose 6-phosphate	0.88
9	Mitochondria	Glucose	0.48*
10	Mitochondria + cell sap	Fructose 6-phosphate	2.58
11	Mitochondria + cell sap	Glucose 6-phosphate	2.37
12	Mitochondria + cell sap	Glucose	1.23

* Exhibited a lag period at low enzyme concentration, but a linear rate was achieved at higher enzyme concentrations in the plot of fructose 1,6-diphosphate formation against enzyme concentration. Since calculation is based on two points only, this value may be in error.

aldolase. This is to be expected, since the mitochondrial fraction contains all the enzymes required for glycolysis in addition to aldolase. The proportion of aldolase in the mitochondria in relation to the other glycolytic enzymes is somewhat greater than that of the cell sap. Aldolase does not appear to be rate-limiting in the mitochondrial fraction; the addition of this enzyme to glycolysing mitochondria did not stimulate the rate of formation of lactate. In the work of Brunngraber & Abood (1960), added aldolase stimulated the glycolytic activity of the cell sap to the same extent as that of added mitochondria. The relatively crude (40% pure) hexokinase used may have contained enzymes (not aldolase) that were rate-limiting. Another explanation for variations in the amount of stimulation in different experiments may reside in the fact that the 'optimum hexokinase concentration' and 'optimum aldolase concentration' may differ with differing rat-brain preparations. Since both an excess and a deficiency of added enzyme gives less than maximum activity, a difference in the enzyme patterns of the rat-brain preparations may cause different effects from time to time. On the other hand, in this, as in the work of Brunngraber & Abood (1960), added aldolase always caused a stimulation of glycolysis.

The inactivation of glycolytic activity at low enzyme concentrations reported by Johnson (1962) was not observed in the present experiments. However, Johnson's (1962) experiments, for the

most part, were conducted with the inclusion of pyruvate in the glycolytic medium. The rate of formation of lactate plotted against time gave a linear relationship, and did not show the characteristic 'lag periods' observed when fructose 1,6-diphosphate and glucose served as the substrates. It is possible that the effects observed by Johnson (1962) were due to the presence of inhibitors in the pyruvate used, or to the pyruvate itself. Johnson (1962) reported inactivation of glycolysis with fructose 1,6-diphosphate as the substrate, but gave no experimental results for this case. The existence of an 'initial lag period' in the lactate formation-time curves with fructose 1,6-diphosphate and glucose as substrates was demonstrated by Brunngraber & Abood (1960). This lag period is exaggerated when low enzyme concentrations are used. It is necessary to determine the slope of the linear portion of the curve to obtain the true rate. Equation of the amount of lactate formed at a single time-interval with the rate of accumulation of lactate is not valid in this case. The existence of a lag period in the kinetics of a multi-enzyme system is not unexpected. This concept has had useful consequences in the analysis of unknown multi-enzyme systems (Bernstein & McGilvery, 1952).

The addition of glutathione to the glycolytic medium inhibited, rather than activated, glycolysis. This does not agree with the results reported by Johnson (1962). Further, the fact that fructose 1,6-diphosphate accumulates in a glycolysing

system supplemented with added hexokinase with glucose as the substrate indicates that the objection raised by Johnson (1961), that our glycolytic medium contains an inadequate amount of fructose 1,6-diphosphate when glycolysis is measured in systems including this compound as substrate, is also without foundation.

The mitochondrial fraction is a heterogeneous mixture of particles containing myelin fragments, nerve endings and synaptic vesicles, in addition to the mitochondria (Whittaker, 1959). The fact that Johnson (1960) has shown lactate dehydrogenase to be more plentiful in particulate fractions that have low oxidative ability, and that Beattie, Sloan & Basford (1962) have prepared, from ox brain, mitochondria that are free of glycolytic activity, suggests that glycolysis may not be a function of the mitochondria at all. Recent work in this Laboratory has indicated that aldolase, glyceraldehyde 3-phosphate dehydrogenase, phosphopyruvate kinase, enolase and phosphopyruvate kinase are richer in those particles of the rat-brain 'mitochondrial fraction' that have low oxidative ability. Hexokinase, however, appears to be located in the mitochondria (Johnson, 1960). Any deductions on the role of 'mitochondrial' glycolysis perhaps should be postponed until the true location of these enzymes in subcellular structures is determined.

The present work has shown that, although aldolase is a rate-limiting enzyme, this limitation is apparent only in systems supplemented with exogenous hexokinase. The hexokinase reaction itself and the availability of ADP appear to be the rate-determining factors in the absence of added hexokinase. These two functions appear to be in balance, so that in the cell sap-plus-mitochondria system no fructose 1,6-diphosphate or triose phosphates will accumulate. At this time the ATP:ADP-plus-AMP ratio is 4.1. Since the addition of hexokinase will double the glycolytic rate, lower the ATP:ADP-plus-AMP ratio to 1.2 and cause accumulation of fructose 1,6-diphosphate, one may speculate whether similar events may not occur in the nervous tissue. An activation of hexokinase and of adenosine triphosphatase, a simultaneous stimulation of adenosine triphosphatase and glycogen (glucose 1-phosphate) utilization or a sudden increase in ATP utilization may expose aldolase as a rate-limiting enzyme and cause fructose 1,6-diphosphate concentrations to rise above the normal levels.

SUMMARY

1. The addition of aldolase stimulated glycolysis by rat-brain cell sap or cell sap supplemented

with mitochondria when the incubation was carried out in the presence of added hexokinase.

2. The fructose 1,6-diphosphate:triose phosphates ratio in rat-brain glycolysis systems in the presence of added hexokinase and glucose as substrates ranged from 5 to 15. The addition of aldolase decreased the rate of accumulation of fructose 1,6-diphosphate by 80–90 %, but increased the rate of accumulation of triose phosphates by 150–350 %. The ratio became 0.5–1.0.

3. The rates of formation of pyruvate from 3-phosphoglycerate and of fructose 1,6-diphosphate from glucose 6-phosphate exceeded the overall glycolytic rates achieved by these preparations. The findings summarized in (1), (2) and (3) provide evidence that aldolase is rate-limiting in these systems when they are supplemented with exogenous hexokinase.

4. In the absence of added hexokinase the rate-limiting factor in both the cell sap and cell sap-plus-mitochondria glycolytic systems appears to be the hexokinase reaction and the availability of ADP. In both systems the addition of hexokinase substantially increased the rate of glycolysis and increased the fructose 1,6-diphosphate:triose phosphates ratio from 2 to 7.5 for the cell sap and from 1.5–2.5 to 5.3 for the cell sap-plus-mitochondria system, as aldolase became rate-limiting.

5. The ATP:ADP-plus-AMP ratios for the cell sap, cell sap-plus-hexokinase, cell sap-plus-mitochondria and cell sap-plus-hexokinase-plus-mitochondria glycolytic systems were 21, 0.56, 4.1 and 1.2 respectively. Aldolase was rate-limiting when the ratios were 1.2 and 0.56; the availability of ADP was rate-limiting when the ratios were 4.1 and 21.

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