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Factors affecting the Glucose 6-Phosphate Inhibition of Hexokinase from Cerebral Cortex Tissue of the Guinea Pig

BY E. A. NEWSHOLME, F. S. ROLLESTON* AND KAY TAYLOR
Department of Zoology, University of Oxford, and Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, University of Oxford

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1. The inhibition of hexokinase by glucose 6-phosphate has been investigated in crude homogenates of guinea-pig cerebral cortex by using a sensitive radiochemical technique for the assay of hexokinase activity. 2. It was observed that 44% of cerebral-cortex hexokinase activity did not sediment with the microsomal or mitochondrial fractions (particulate fraction), and this is termed soluble hexokinase. The sensitivities of soluble and particulate hexokinase, and hexokinase in crude homogenates, to the inhibitory actions of glucose 6-phosphate were measured; 50% inhibition was produced by 0·023, 0·046 and 0·068mM-glucose 6-phosphate for soluble, particulate and crude homogenates respectively. 3. The optimum Mg^{2+} concentration for the enzyme was about 10mM, and this appeared to be independent of the ATP concentration. In the presence of added glucose 6-phosphate, raising the Mg^{2+} concentration to 5mM increased the activity of hexokinase, but above this concentration Mg^{2+} potentiated the glucose 6-phosphate inhibition. When present at a concentration above 1mM, Ca^{2+} ions inhibited the enzyme in the presence or absence of glucose 6-phosphate. 4. When the ATP/Mg^{2+} ratio was 1·0 or below, variations in the ATP concentration had no effect on the glucose 6-phosphate inhibition; above this value ATP inhibited hexokinase in the presence of glucose 6-phosphate. ATP had an inhibitory effect on soluble hexokinase similar to that on a whole-homogenate hexokinase, so that the ATP inhibition could not be explained by a conversion of particulate into soluble hexokinase (which is more sensitive to inhibition by glucose 6-phosphate). It is concluded that ATP potentiates glucose 6-phosphate inhibition of cerebral-cortex hexokinase, whereas the ATP–Mg^{2+} complex has no effect. Inorganic phosphate and L-α-glycerophosphate relieved glucose 6-phosphate inhibition of hexokinase; these effects could not be explained by changes in the concentration of glucose 6-phosphate during the assay. 5. The inhibition of hexokinase by ADP appeared to be independent of the glucose 6-phosphate effect and was not relieved by inorganic phosphate. 6. The physiological significance of the ATP, inorganic phosphate and α-glycerophosphate effects is discussed in relation to the control of glycolysis in cerebral-cortex tissue.

Weil-Malherbe & Bone (1951) first showed that hexokinase (EC 2.7.1.1) in crude extracts of rat brain was inhibited by glucose 6-phosphate non-competitively with respect to glucose; this finding was confirmed with a partially purified preparation of brain hexokinase by Crane & Sols (1953). The detailed kinetics of glucose 6-phosphate inhibition of brain hexokinase were studied by Fromm & Zewe (1962), who used a coupled enzyme assay to follow ADP formation. They showed that the inhibition was uncompetitive with respect to glucose. The ability of P_i to relieve glucose 6-phosphate inhibition of brain hexokinases was first reported by Tiedemann & Born (1959), and subsequently by Rose & O'Connell (1964) with erythrocyte hexokinase. However, Lowry & Passonneau (1964) observed no effect of P_i on glucose 6-phosphate inhibition of mouse brain hexokinase, and S. Rapoport & G. Gerber (unpublished work cited by Walker, 1966) found no effect of inorganic phosphate on partially purified human erythrocyte hexokinase, which contained no phosphofructokinase activity. Thus it has been suggested that the relief of glucose 6-phosphate inhibition of hexokinase by P_i may be explained by a lowering of the concentration of glucose 6-phosphate through P_i

* Present address: Department of Physiology, University of Chicago, Chicago, Ill. 60637, U.S.A.

activation of phosphofructokinase. The absence of a P_i effect when phosphofructokinase was removed from the system by purification is in accord with this possibility (see Walker, 1966). However, loss of the effect of P_i could also be explained by the purification procedures destroying the sensitivity of the hexokinase to P_i, but not to glucose 6-phosphate.

One problem in the investigation of glucose 6-phosphate inhibition of hexokinase is the method of assay of the activity of the enzyme in the presence of glucose 6-phosphate. The earlier workers and Lowry & Passonneau (1964) followed glucose disappearance, but this method lacks sensitivity and accuracy; if ADP formation is followed with a coupled assay system, hexokinase has to be purified so that contaminating enzymes (e.g. non-specific adenosine triphosphatase and adenylate kinase) are removed. In the present work a simple and sensitive radiochemical technique for measuring hexokinase activity (Newsholme, Robinson & Taylor, 1967) has been used to investigate factors that might modify the glucose 6-phosphate inhibition of hexokinase in crude homogenates of cerebral cortex of the guinea pig. The assay depends upon the incorporation of [^{14}C]glucose into glucose 6-phosphate and separation of these on DEAE-cellulose paper disks.

The enzyme in cerebral cortex tissue was selected for investigation because Rolleston & Newsholme (1967) have shown that glucose 6-phosphate inhibition of hexokinase plays an important role in the regulation of glycolysis in this tissue. The results reported show that ATP, α-glycerophosphate and P_i can modify the glucose 6-phosphate inhibition of hexokinase, and the physiological significance of these findings is discussed.

MATERIALS AND METHODS

Animals and materials. Adult guinea pigs were used as described by Rolleston & Newsholme (1967). Chemicals and enzymes were obtained from the sources described by Newsholme *et al.* (1967). [U-^{14}C]Glucose (specific radioactivity 100–200 mc/m-mole) was obtained from The Radiochemical Centre, Amersham, Bucks.

Preparation of crude extracts. The brain of a guinea pig killed by cervical dislocation was removed and placed into ice-cold 0·9% NaCl. The cerebral-cortex grey matter was dissected out, weighed and homogenized in 50 vol. of 10mM-triethanolamine–HCl, pH 7·0, in a manually operated glass Potter–Elvehjem homogenizer (clearance approx. 0·025 cm.). This homogenate was used for hexokinase activity measurements without any centrifugation or further treatment. Generally, 20μl. of this homogenate was used for hexokinase activity determinations; this represents about 0·4 mg. of cerebral-cortex tissue or 0·034 mg. of protein.

Cell fractionation. The cerebral-cortex grey matter was homogenized in 10 vol. of 0·3M-sucrose–2mM-EDTA–10mM-triethanolamine–HCl, pH 7·2, in a manually operated Potter–Elvehjem homogenizer (clearance approx. 0·025 cm.). Mitochondria (plus nerve endings), microsomes and supernatant fraction were separated according to the method of Gray & Whittaker (1962). The mitochondrial, microsomal and cell-debris pellets were resuspended in 5, 3 and 1 ml. respectively of the above homogenizing medium.

Assay of hexokinase activity. Hexokinase activity was assayed by the radiochemical method described by Newsholme *et al.* (1967). The incubation medium usually consisted of 100mM-triethanolamine–HCl, 0·17mM-[1-^{14}C]-glucose (specific radioactivity approx. 10 mc/m-mole) and various concentrations of ATP and Mg^{2+}, pH 7·4. A sample (20μl.) of cerebral-cortex homogenate was incubated with 100μl. of the incubation medium at 28° (or at room temperature). After incubation the reaction was stopped by the addition of 100μl. of 98% ethanol. Samples (20μl.) of this solution were pipetted on to disks of DEAE-cellulose paper, which were washed, dried and counted as described by Newsholme *et al.* (1967), except that in the present investigation the paper disks were counted in a Beckman liquid-scintillation spectrometer, model 1650.

Hexokinase activities reported in this paper are based on counts retained on the paper after 3 or 5 min. periods of incubation. A zero-time count rate (obtained by addition of ethanol to incubation medium before the homogenate) is always subtracted from the count rate at these times of incubation; this represents the small percentage of [^{14}C]-glucose that adheres to the DEAE-cellulose paper disks (see Newsholme *et al.* 1967). Preliminary experiments showed that at higher concentrations of homogenate the progress curve was not linear and decreased with time. This decrease in activity could be correlated with the accumulation of glucose 6-phosphate during the assay, and it could be minimized by lowering the concentration of homogenate and the incubation time (under these conditions it was necessary to increase the specific radioactivity of the [^{14}C]glucose in the incubation medium). It was not possible to avoid completely this non-linearity in the progress curve, but for all activities reported in this paper the increase in glucose 6-phosphate concentration during incubation (3 or 5 min. duration) could result in not more than 10% inhibition of hexokinase. There was no conversion of glucose into glucose 6-phosphate, as determined radiochemically, in the absence of ATP.

The addition of standard [^{14}C]glucose 6-phosphate (0·07–0·17mM) to a mixture of the homogenate and incubation medium (minus [^{14}C]glucose) could be completely recovered when assayed radiochemically after 3 min. incubation at 25°. The estimation of this glucose 6-phosphate by enzymic methods for glucose 6-phosphate plus fructose 6-phosphate resulted in recoveries of 85% of the added glucose 6-phosphate; no allowance is made for this loss in the concentrations of glucose 6-phosphate reported in this paper.

Glucose 6-phosphate and fructose 6-phosphate were measured enzymically by the method of Newsholme & Randle (1961), α-glycerophosphate by the method of Hohorst (1963) and ADP by the method of Adam (1963).

Expression of results. Activities are usually expressed as counts/min. retained on the DEAE-cellulose paper multiplied by a factor (usually 11), which represents the ratio of sample taken to the total volume of inhibited incubated mixture. Thus the values presented provide some indication of the formation of radioactive glucose 6-phosphate

in the incubation tube. All activity measurements were made in duplicate with a reproducibility of 10%; each experiment reported is representative of at least two separate experiments.

From a knowledge of the amount of glucose in the incubation tube (0·02 μmole), the total number of counts/min. in the incubation tube (270 000–330 000) and the amount of tissue used (usually 1/2500 g.) the activity in counts/min. incorporated into product/min. of incubation time can be converted into μmoles of product formed/min./g. fresh wt. of tissue (see legend to Fig. 1).

RESULTS

The activities of hexokinase in various cell fractions of cerebral cortex are shown in Table 1: approximately equal activities of hexokinase were found in the particulate (mitochondria plus microsomes etc.) and the soluble fraction. For this reason the sensitivities of the soluble and particulate hexokinases to glucose 6-phosphate inhibition were investigated and compared with the sensitivity in the whole homogenate; the results are reported in Fig. 1. The soluble hexokinase appears to be more sensitive to glucose 6-phosphate inhibition, particularly at lower concentrations of the inhibitor. The inhibition of activity in whole homogenate is similar to that of the particulate fraction. Thus 50% inhibition is produced by approx. 0·023, 0·046 and 0·068 mM-glucose 6-phosphate for soluble, particulate and whole homogenate respectively. The reason for these differences in sensitivity to glucose 6-phosphate inhibition is not known, and it is possible that it reflects an experimental condition that may be of little physiological significance.

The glucose 6-phosphate inhibition of hexokinase in crude homogenates was reversed by removal of the glucose 6-phosphate (by the addition to the incubation of NADP and glucose 6-phosphate dehydrogenase); this confirms the early findings of Crane & Sols (1953).

A large number of compounds were tested to see if they would modify the glucose 6-phosphate

Table 1. *Hexokinase activity in cell fractions of cerebral cortex*

The cell fractions were prepared as described by Gray & Whittaker (1962) (see also Fig. 1) and P_1, P_2 and P_3 refer to the fractions described by Gray & Whittaker (1962). Hexokinase activity was assayed by following the reduction of NADP at 340 mμ with a Gilford recording spectrophotometer at 25°. The incubation buffer was similar to that for the radiochemical assay except that unlabelled glucose was used and 0·1 mM-NADP and glucose 6-phosphate dehydrogenase (0·1 mg./2 ml. of incubation buffer) were added. The concentrations of ATP, Mg^{2+} and glucose were 1, 2 and 0·5 mM respectively.

Cell fraction	Hexokinase activity (μmoles/min./g. fresh wt. of original tissue)	Hexokinase activity (% of whole homogenate)
Whole homogenate	3·22	—
Cell debris and nuclei (P_1)	0·19	6
Mitochondria and nerve endings (P_2)	1·49	46
Microsomes (P_3)	0·52	16
Soluble fraction	1·43	44

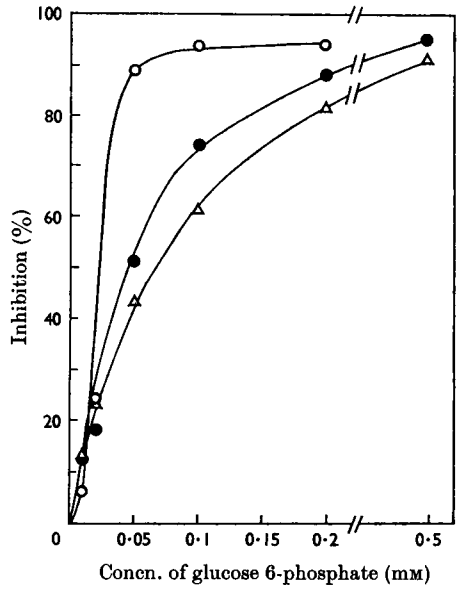

Fig. 1. Percentage inhibition of whole-homogenate, particulate and soluble hexokinases of cerebral cortex by glucose 6-phosphate. The cerebral cortex of a guinea pig was homogenized in 10 vol. of 0·32 M-sucrose, and centrifuged at 1000*g* for 10 min. The precipitate was suspended in 24 ml. of sucrose and centrifuged again. The supernatant and washings were centrifuged at 30000*g* at 0° for 55 min. The precipitate was suspended in 5 ml. of sucrose and diluted fivefold with sucrose before assay; this precipitate contains both the mitochondrial and the microsomal fraction of Table 1 (P_2 and P_3), and is termed the particulate fraction. The supernatant, which is termed the soluble fraction, was used for assay without dilution. The whole homogenate consisted of the cortex homogenized in sucrose but without any centrifugation. Hexokinase activity was measured by the radiochemical method (see the Materials and Methods section); the concentrations of glucose, ATP, Mg^{2+} and glucose 6-phosphate were 0·17, 1·0, 1·0 and 0·1 mM respectively. ○, Soluble fraction; ●, particulate fraction; △, whole homogenate. The activities of hexokinase in the absence of added glucose 6-phosphate for whole homogenate, particles and soluble fraction were 0·75, 0·42 and 0·21 μmole/min./g. wet wt. of cortex or 116300, 68700 and 33000 counts/min. incorporated into product/5 min. incubation/20 μl. of extract respectively.

inhibition of hexokinase in whole homogenates. The following compounds, which were tested in the range 1–10mM, had no significant effect on glucose 6-phosphate inhibition: AMP, fructose diphosphate, citrate, isocitrate, glutamate, aspartate, malate, phosphoenolpyruvate, NAD, NADP, $NADH_2$. However, P_i and α-glycerophosphate lowered the inhibition, whereas ATP increased it; these compounds had little or no effect in the absence of glucose 6-phosphate; but Mg^{2+} and Ca^{2+} ions modified hexokinase activity in the presence or absence of glucose 6-phosphate. The effects of ATP, P_i, α-glycerophosphate, Mg^{2+} and Ca^{2+} were investigated in more detail.

Effect of Mg^{2+} *and* Ca^{2+} *ions.* In the initial studies on this enzyme the Mg^{2+}/ATP ratio was maintained at 1:1 or 2:1 (see Fromm & Zewe, 1962; Grossbard & Schimke, 1966), so that the final concentration of Mg^{2+} was usually 1 or 2mM. However, it was observed that raising the Mg^{2+} concentration above this ratio increased the activity of hexokinase (Table 2). The optimum Mg^{2+} concentration appears to be about 10mM, and this was not affected by raising the ATP concentration from 1 to 2mM. In the presence of added glucose 6-phosphate a similar increase in activity was observed up to 5mM-Mg^{2+}; above this concentration, Mg^{2+} inhibited the activity (Table 2); it is suggested that this represents a potentiation of the glucose 6-phosphate inhibition.

Low concentrations of Ca^{2+} (up to 1·0mM) increased the activity of hexokinase, but concentrations above this decreased the activity very markedly either in the presence or absence of added glucose 6-phosphate (Table 3). This effect is of interest as Lowry & Passonneau (1966) and Underwood & Newsholme (1967) have reported that brain and kidney phosphofructokinase (respectively) are inhibited by Ca^{2+}.

Effects of P_i *and α-glycerophosphate.* P_i or α-glycerophosphate slightly increased the activity of hexokinase in the absence of added glucose 6-phosphate (increase of 14%), but they markedly increased the activity in the presence of added glucose 6-phosphate (Table 4). The increase in activity in the absence of added glucose 6-phosphate may be explained by P_i or α-glycerophosphate relieving the inhibition of hexokinase caused by the small accumulation of glucose 6-phosphate during the incubation period (see the Materials and Methods section). Although concentrations of these compounds as low as 0·5mM lowered the inhibition

Table 3. *Effect of* Ca^{2+} *ions on the activity of hexokinase in the presence and absence of added glucose 6-phosphate*

Hexokinase activity was measured by the radiochemical method (see the Materials and Methods section) and the concentrations of ATP, Mg^{2+} and glucose were 1, 1, and 0·17mM respectively.

Concn. of Ca^{2+} (mM)	10^{-2} × Hexokinase activity (counts/min. incorporated into product/3min. incubation/20μl. of homogenate): Absence of added glucose 6-phosphate	Presence of 0·1mM-glucose 6-phosphate
0	315	42
0·5	—	53
1·0	345	49
2·0	222	37
5·0	157	25
10·0	15	—

Table 2. *Effect of* Mg^{2+} *ions on hexokinase activity in the presence and absence of glucose 6-phosphate*

Hexokinase activity was measured by the radiochemical method (see the Materials and Methods section); the concentration of ATP was 1mM, glucose 0·17mM and Mg^{2+} as shown. The units of hexokinase activity are counts/min. incorporated into product/3min. incubation/20μl. of homogenate.

Concn. of Mg^{2+} (mM)	Hexokinase activity: Absence of added glucose 6-phosphate: 10^{-2} × Radioactivity incorporated (units)	Absence of added glucose 6-phosphate: Increase over 1mM-Mg^{2+} (%)	Presence of 0·07mM-glucose 6-phosphate: 10^{-2} × Radioactivity incorporated (units)	Presence of 0·07mM-glucose 6-phosphate: Increase over 1mM-Mg^{2+} (%)
1	359	—	135	—
2	506	41	150	11
5	554	55	201	49
10	624	74	178	31
15	600	67	141	4
20	642	79	128	−5

Table 4. *Effect of P_i and L-α-glycerophosphate on glucose 6-phosphate inhibition of hexokinase*

Hexokinase activity was assayed by the radiochemical method (see the Materials and Methods section) and the concentrations of glucose, ATP and Mg^{2+} were 0·17, 1 and 1 mM respectively.

Addition	Concn. (mM)	10^{-2} × Hexokinase activity (counts/min. incorporated into product/3 min. incubation/20 μl. of homogenate)		Inhibition by glucose 6-phosphate (%)
		Absence of added glucose 6-phosphate	Presence of 0·1 mM-glucose 6-phosphate	
None	—	418	78	81
P_i	0·25	378	91	76
	0·5	379	108	71
	1·0	427	132	69
	5·0	464	238	49
	10·0	476	269	43
	20·0	457	277	39
L-α-Glycerophosphate	0·2	401	82	80
	0·5	424	95	77
	1·0	443	96	78
	2·5	457	131	71
	5·0	455	157	66
	10·0	458	170	63

slightly, it was not completely removed by concentrations of 10 or 20 mM. It was considered impractical to increase the concentrations of these compounds more than this because they might have interfered with the binding of [^{14}C]glucose 6-phosphate to the DEAE-cellulose paper (see Newsholme *et al.* 1967).

In a number of experiments the concentration of glucose 6-phosphate at the end of the incubation was measured by stopping the reaction with perchloric acid (instead of alcohol), neutralizing with potassium hydrogen carbonate and estimating glucose 6-phosphate enzymically. The concentration of glucose 6-phosphate was found to be unchanged by the presence of P_i or of α-glycerophosphate. However, as the total amount of glucose 6-phosphate in the incubation was small (approx. 0·01 μmole) it was difficult to measure, even though a Gilford recording spectrophotometer at maximum sensitivity was used. To obtain more reliable glucose 6-phosphate measurements, the volumes of homogenate and incubation buffer were increased in proportion and the incubation was carried out for the same period and the reaction stopped with perchloric acid; P_i and α-glycerophosphate again had no effect on the concentrations of glucose 6-phosphate (Table 5).

In a similar experiment the concentration of α-glycerophosphate was measured enzymically and it was found that α-glycerophosphate was not utilized during the incubation. Thus the effect of α-glycerophosphate on glucose 6-phosphate inhibition could not be explained by hydrolysis to provide P_i.

Effects of ATP. At 1 mM-Mg^{2+} and in the absence of added glucose 6-phosphate, maximum activity of hexokinase was observed at about 2 mM-ATP, and ATP was not inhibitory; however, in the presence of added glucose 6-phosphate, ATP above 1 mM inhibited hexokinase activity (Fig. 2*a*). When the concentration of Mg^{2+} was increased to 15 mM, ATP was not inhibitory (Fig. 2*b*). It has previously been shown that glucose 6-phosphate inhibition of purified brain hexokinase is competitive with ATP–Mg^{2+} (Fomm & Zewe, 1962; Grossbard & Schimke, 1966). In the present investigation increasing the ATP concentration either had no effect on glucose 6-phosphate inhibition or it increased the inhibition.

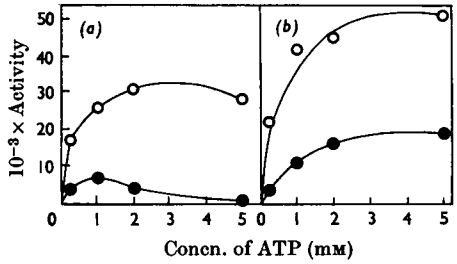

Fig. 2. Effect of ATP on hexokinase activity at two Mg^{2+} concentrations in the presence and absence of added glucose 6-phosphate. Hexokinase activity was measured by the radiochemical method (see the Materials and Methods section); glucose concentration was 0·17 mM. Hexokinase activity is presented as counts/min. incorporated into product/3 min. incubation/20 μl. of homogenate. (*a*) Mg^{2+} concentration 1 mM; (*b*) Mg^{2+} concentration 15 mM. ○, No addition of glucose 6-phosphate; ●, 0·1 mM-glucose 6-phosphate.

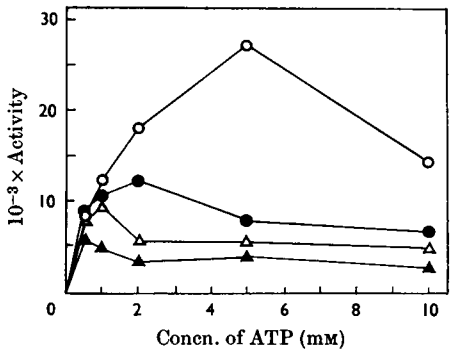

Fig. 3. Effect of ATP concentrations on hexokinase activity in the presence of an inhibitory concentration of glucose 6-phosphate and varying Mg^{2+} concentrations. Hexokinase was assayed by the radiochemical method (see the Materials and Methods section) and is presented as counts/min. incorporated into product/3min. incubation/20μl. of homogenate; the concentrations of glucose and glucose 6-phosphate were 0·17 and 0·1mM respectively. Concentrations of Mg^{2+} and ATP were varied. ▲, 0·5mM-Mg^{2+}; △, 1mM-Mg^{2+}; ●, 2·0mM-Mg^{2+}; ○, 5mM-Mg^{2+}.

Table 6. *Effect of ATP on soluble and whole-homogenate hexokinase activities in the presence of glucose 6-phosphate*

The soluble fraction and whole homogenate were prepared as described in Fig. 1. The concentrations of glucose, glucose 6-phosphate and Mg^{2+} were 0·17, 0·07 and 2mM respectively. At 2mM-ATP–Mg^{2+}, in the absence of added glucose 6-phosphate, the counts/min. incorporated into product/3min. incubation/20μl. of preparation were 22100 and 9410 for whole homogenate and soluble fraction respectively.

Concn. of ATP (mM)	Hexokinase activity (counts/min. incorporated into product/3min. incubation/20μl. of preparation)		Inhibition above 2mM-ATP (%)	
	Whole homogenate	Soluble fraction	Whole homogenate	Soluble fraction
1	5390	720	—	—
2	6440	2480	—	—
5	4230	1920	34	24
10	3320	1540	49	38
20	2790	1340	57	46

Table 5. *Effect of ATP, P_i and L-α-glycerophosphate on the concentration of glucose 6-phosphate added to the incubation medium*

The incubation medium was the same as that used for radiochemical assay of hexokinase except that unlabelled glucose was used, and the volumes of homogenate and incubation buffer were increased to 0·1ml. and 0·5ml. respectively. Incubation was carried out for 3min. before addition of $HClO_4$. The final concentration of glucose 6-phosphate in the incubation was 0·1mM and the concentration of ATP–Mg^{2+} was 1mM unless otherwise stated.

Addition to incubation	Concn. after incubation (mM)	
	Glucose 6-phosphate	ADP
—	0·10	0·23
P_i (5mM)	0·10	—
P_i (10mM)	0·10	—
ATP (5mM)	0·10	0·43
ATP–Mg^{2+} (5mM)	0·11	0·43
L-α-Glycerophosphate (5mM)	0·11	—

No explanation for this difference is offered, but it is pointed out that in the present work the concentration of ATP, not the concentration of ATP–Mg^{2+}, was varied.

The effects of varying both the ATP and Mg^{2+} concentrations are shown in Fig. 3. ATP was inhibitory only when the ATP/Mg^{2+} ratio exceeded 1·0, suggesting that ATP and not the ATP–Mg^{2+} complex potentiated the glucose 6-phosphate inhibition of hexokinase. The possibility that ATP inhibition was due to an accumulation of either glucose 6-phosphate or ADP, which is also an inhibitor of hexokinase, was excluded by measuring the concentrations of glucose 6-phosphate and ADP after incubation with inhibitory and non-inhibitory concentrations of ATP. The concentrations of glucose 6-phosphate and ADP were almost identical in the two conditions (Table 5).

The effects of inhibitory concentrations of ATP on the activity of soluble and whole homogenate hexokinase (in the presence of glucose 6-phosphate) are shown in Table 6. ATP inhibited both hexokinases, the soluble one being slightly less sensitive to the inhibitor.

Inhibition of phosphofructokinase by ATP could be potentiated by citrate and relieved by AMP, fructose diphosphate, P_i and 3,5-(cyclic)-AMP (Passonneau & Lowry, 1962; Mansour, 1963). It was therefore decided to ascertain whether these compounds affected the ATP potentiation of glucose 6-phosphate inhibition of hexokinase. Except for P_i, these compounds had no effect on hexokinase activity in the presence of inhibitory concentrations of ATP. The experiment reported in Fig. 4 showed that P_i relieved both the glucose 6-phosphate and the ATP inhibitions of hexokinase, as might be expected if ATP acts by potentiating the glucose 6-phosphate inhibition.

Effects of ADP and glucose 6-phosphate. ADP is also an inhibitor of brain hexokinase (Sols & Crane, 1954; Fromm & Zewe, 1962). Most investigators

have studied the inhibitory effects of one product by following the formation of the second product with a coupled enzymic assay, but it has not been possible to study the effects of both products simultaneously. The radiochemical assay used in this work permits such an investigation. The presence of inhibitory concentrations of ADP did not modify the inhibition of hexokinase by glucose 6-phosphate (Table 7). The two inhibitory actions appear to be independent, so that their effects are additive.

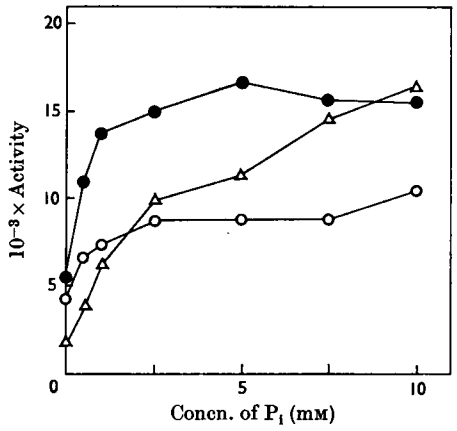

Fig. 4. Effect of inorganic phosphate on glucose 6-phosphate, ATP and ADP inhibitions of hexokinase. Hexokinase was assayed by the radiochemical method (see the Materials and Methods section) and is presented as counts/min. incorporated into product/3min. incubation/20μl. of homogenate. The concentrations of ATP, Mg^{2+} and glucose were 1, 1 and 0·17mM respectively, except in one experiment in which the ATP concentration was increased to 5mM, but Mg^{2+} remained at 1mM. ●, 0·1mM-Glucose 6-phosphate was added to incubation medium; △, 0·1mM-glucose 6-phosphate was added and the ATP concentration was raised to 5mM; ○, 0·1mM-glucose 6-phosphate and 2·0mM-ADP were added to the incubation medium.

Fig. 4 also shows the effect of P_i on ADP inhibitions of hexokinase. Although P_i relieved glucose 6-phosphate inhibition, it had no effect on the inhibition of hexokinase by ADP, suggesting that the inhibitions of hexokinase by glucose 6-phosphate and ADP were independent.

DISCUSSION

Hexokinase has been shown to be a regulatory enzyme for glycolysis in cerebral cortex (Rolleston & Newsholme, 1967), and the inhibition of this enzyme by glucose 6-phosphate is a property on which a theory of metabolic control of glycolysis is based (for review see Walker, 1966). The earlier work of Crane & Sols (1954) established that the glucose 6-phosphate inhibition was very specific for this sugar phosphate, and Lowry & Passonneau (1964) showed that a number of other metabolic intermediates had little or no effect on this enzyme. However, the question has been raised whether the inhibition by glucose 6-phosphate could be modified by other metabolic intermediates, such as P_i, in a similar manner to the relief of ATP inhibition of phosphofructokinase by AMP, fructose diphosphate etc.

Apart from the effect of P_i, this problem seems to

Table 7. *Inhibition of hexokinase by ADP and glucose 6-phosphate*

Hexokinase activity was measured by the radiochemical method (see the Materials and Methods section); the concentrations of ATP, Mg^{2+} and glucose were 5, 5, and 0·17mM respectively. ADP and glucose 6-phosphate were added at the concentrations indicated.

Concn. of ADP (mM)	Concn. of glucose 6-phosphate (mM)	10^{-2} × Hexokinase activity (counts/min. incorporated into product/3min. incubation/20μl. of homogenate)	Inhibition of hexokinase (%)	Inhibition due to glucose 6-phosphate (%)
0	0	419	0	0
0	0·025	320	24	24
0	0·1	122	70	70
0·5	0	354	15	0
0·5	0·025	262	37	26
0·5	0·1	90	78	74
1·0	0	328	22	0
1·0	0·025	264	37	20
1·0	0·1	90	78	72
2·0	0	300	28	0
2·0	0·025	235	44	22
2·0	0·1	79	81	74

have been generally neglected, probably because of the difficulties in measuring accurately glucose 6-phosphate inhibition of hexokinase in crude extracts (see the introduction). Detailed investigations of glucose 6-phosphate inhibition of hexokinase have been carried out by following the formation of ADP in a coupled enzyme system; this requires some purification of the hexokinase, which could result in distortion or removal of regulatory properties. The radiochemical assay used in the present work is simple and very sensitive. The crude brain homogenate used in these assays could be diluted to such an extent that ions, cofactors or metabolic intermediates that might interfere in the assay were reduced to negligible concentrations (concentrations of glucose 6-phosphate and NADP in the final incubation mixture were 10 and 2 μM respectively).

The high optimum concentration of Mg^{2+} for this enzyme, under these conditions, has not been reported previously. Other investigators have used a specific ATP/Mg^{2+} ratio, but do not state whether the Mg^{2+} concentration was optimum for hexokinase activity (e.g. see Fromm & Zewe, 1962). Another effect of Mg^{2+} (above 5mM) was to increase the inhibition by glucose 6-phosphate; it seems possible that at low concentrations of glucose 6-phosphate these two effects of Mg^{2+} (namely an increase in enzyme activity and potentiation of glucose 6-phosphate inhibition) could cancel each other out, and produce an apparent constant activity with increasing Mg^{2+} concentrations.

The other factors that changed glucose 6-phosphate inhibition were ATP, P_i and α-glycerophosphate. When the ratio ATP/Mg^{2+} exceeded 1·0, ATP produced inhibition of hexokinase; as this effect was observed only in the presence of glucose 6-phosphate it is concluded that ATP potentiates the glucose 6-phosphate inhibition. On the other hand, P_i and α-glycerophosphate increased hexokinase activity, but only in the presence of glucose 6-phosphate; it is concluded that these compounds relieve the glucose 6-phosphate inhibition of hexokinase. It must be stressed that these effects cannot be explained by changes in the glucose 6-phosphate concentration during incubation. However, the mechanism by which these compounds modify the glucose 6-phosphate inhibition is unknown: they could, for example, change the structural configuration of hexokinase so that its sensitivity to glucose 6-phosphate is modified, either by binding directly to the enzyme, or by affecting the rate of interconversion between two forms of hexokinase, which differ in sensitivity to glucose 6-phosphate.

Rose & Warms (1967) have shown with ascites-tumour hexokinase that ATP causes the conversion of particulate into soluble hexokinase. As soluble hexokinase of the cerebral cortex is more sensitive to glucose 6-phosphate inhibition (Fig. 1), the observed inhibitory action of ATP with whole homogenate could be explained by conversion of particulate into soluble hexokinase. However, as soluble hexokinase was inhibited by ATP (Table 6), this explanation is unlikely.

The physiological significance of these effects of ATP, α-glycerophosphate and P_i is uncertain. Lowry, Passonneau, Hasselberger & Schulz (1964) have shown that ischaemia, which increases glycolysis, lowers the ATP and glucose 6-phosphate concentrations and raises that of P_i in whole mouse brain. Rolleston & Newsholme (1967) have shown that factors that increase glycolysis in cerebral-cortex slices decrease ATP and glucose 6-phosphate concentrations, although that of P_i was usually unchanged. It seems probable that control of hexokinase depends primarily on glucose 6-phosphate inhibition, but that the effect of changes in glucose 6-phosphate concentration can be magnified through changes in the concentrations of ATP, P_i or both. This would amplify the effect of the change in the glucose 6-phosphate concentration on the hexokinase activity; because of the actions of ATP and P_i a smaller change in the concentration of glucose 6-phosphate is required to elicit a maximum response from hexokinase. Such amplification may be necessary because a fall in the concentration of glucose 6-phosphate required to stimulate hexokinase could seriously lower the concentration of substrate (fructose 6-phosphate) required for phosphofructokinase; this may be particularly important as the ATP inhibition of phosphofructokinase is related to the concentration of fructose 6-phosphate in a competitive manner (Passonneau & Lowry, 1962; Underwood & Newsholme, 1965, 1967).

The physiological significance of the α-glycerophosphate relief of glucose 6-phosphate inhibition is questionable; thus the intracellular concentration is low (0·3mM) in comparison with the concentrations required to produce marked relief of inhibition (Table 4). However, the α-glycerophosphate concentration in cerebral-cortex slices is not dependent upon the $NADH_2$/NAD ratio as in other tissues (Rolleston & Newsholme, 1967); it seems possible that the high activity of α-glycerophosphate oxidase in brain may be important in control of the concentration of α-glycerophosphate and therefore in control of hexokinase activity and the glycolytic rate.

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