Biochem. J. (1971) **123**, 707–715
Printed in Great Britain

Cerebral-Cortex Hexokinases

ELUCIDATION OF REACTION MECHANISMS BY SUBSTRATE AND DEAD-END INHIBITOR KINETIC ANALYSIS

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(*Received* 15 *February* 1971)

1. The substrate kinetic properties of cerebral hexokinases (mitochondrial and cytoplasmic) were studied at limiting concentrations of both glucose and $MgATP^{2-}$. Primary plots of the enzymic activity gave no evidence of a Ping Pong mechanism in three types of mitochondrial preparation tested (intact and osmotically disrupted mitochondria, and the purified mitochondrial enzyme), nor in the purified cytoplasmic preparation. 2. Secondary plots of intercepts from the primary plots ($1/v$ versus $1/s$) versus reciprocal of second substrate of the mitochondrial activity gave kinetic constants which differed from those obtained directly from the plots of $1/v$ versus $1/s$ or of s/v versus s, although the ratios of the derived constants were consistent. The kinetic constants obtained with the cytoplasmic enzyme from primary and secondary plots were consistent. 3. Deoxyglucose, as alternative substrate, inhibited cytoplasmic hexokinase by competition with glucose, but did not compete when $MgATP^{2-}$ was the substrate varied. The K_i for deoxyglucose when glucose concentrations were varied was 0.25mM. 4. A range of ATP analogues was tested as potential substrates and inhibitors of hexokinase activity. GTP, ITP, CTP, UTP and $\beta\gamma$-methylene-ATP did not act as substrates, nor did they cause significant inhibition. Deoxy-ATP proved to be almost as effective a substrate as ATP. AMP inhibited but did not act as substrate. 5. *N*-Acetylglucosamine inhibited all preparations competitively when glucose was varied and non-competitively when $MgATP^{2-}$ was varied. AMP inhibition was competitive when $MgATP^{2-}$ was the substrate varied and non-competitive when glucose was varied. 6. The results are interpreted as providing evidence for a random reaction mechanism in all preparations of brain hexokinase, cytoplasmic and mitochondrial. The kinetic properties and reaction mechanism do not change on extraction and purification of the particulate enzyme. 7. The results are discussed in terms of the participation of hexokinase in regulation of cerebral glycolysis.

The reaction mechanism of brain hexokinase is at present uncertain since the evidence available is too conflicting to permit clear distinction between Ping Pong, other sequential mechanisms and a random mechanism. Moreover, although the particulate and cytoplasmic hexokinases show similar K_m values for the two substrates (glucose and $MgATP^{2-}$), derived from primary plots (Thompson & Bachelard, 1970), it is not known if the enzymes from the two subcellular sources have identical reaction mechanisms.

Earlier studies of Fromm & Zewe (1962) on the particulate hexokinase from bovine brain were indicative of a Ping Pong mechanism. This mechanism also appeared to operate in the enzyme after deoxycholate extraction from the particulate fraction (Copley & Fromm, 1967), although pronounced increase in the K_m for ATP was observed in the solubilized preparation. Subsequently, using D-fructose as substrate for the detergent-extracted particulate enzyme, Fromm & Ning (1968) suggested a sequential mechanism other than Ping Pong, based on arguments that glucose and fructose react at the same site on the enzyme. More recently, results from the same laboratory were interpreted in terms of a random mechanism for the solubilized particulate activity (Ning, Purich & Fromm, 1969). Such reports of three different types of reaction mechanism for the same hexokinase preparation serve to illustrate the difficulties which have been

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encountered in establishing its reaction mechanism. No such studies appear to have been made on the cytoplasmic enzyme from the brain.

Cleland (1963*a*,*b*,*c*) has clearly set out a systematic approach for elucidation of the reaction mechanisms of enzymes having two or more substrates and two or more products. The approach is based on study of the kinetics of interaction of substrates and products and emphasis is placed on the necessity, in studies based on initial velocity analysis, for the concentrations of both substrates to be below those required to saturate the enzyme.

A study of the kinetics of inhibition by the products is complicated by the interactions of the adenine nucleotides, ATP and ADP, with Mg^{2+} (Bachelard & Goldfarb, 1969). Since the binding constant of $MgADP^-$ is considerably lower than that of $MgATP^{2-}$, it is difficult to distinguish inhibition by $MgADP^-$ from that by free ADP, unless excessive concentrations of Mg^{2+} are employed. A further point of uncertainty arising from ADP inhibition studies is whether ADP (or $MgADP^-$) acts as a true product inhibitor or as a 'mixed dead-end and product' inhibitor (Cleland, 1963*b*). These reflections emphasize the importance of a clear understanding of the substrate kinetics before intelligent interpretation of the results of product inhibition or dead-end inhibitor kinetic studies can be made.

This paper reports the substrate kinetic properties of cerebral cytoplasmic and mitochondrial hexokinases under conditions of limiting concentrations of the two substrates, glucose and $MgATP^{2-}$. The kinetics for 2-deoxy-D-glucose as alternate substrate are also presented. Two dead-end inhibitors of cerebral hexokinase have been used for elucidation of the sequence of addition of substrates to the enzyme. The results clearly indicate the presence of a random reaction mechanism in the mitochondrial enzyme, both particle-bound and solubilized, and also in the cytoplasmic enzyme.

MATERIALS AND METHODS

Sources of hexokinase. The cytoplasmic hexokinase was the purified major component from DEAE-cellulose columns (peak I, Thompson & Bachelard, 1970). Three preparations of mitochondrial hexokinase were used: (*a*) intact mitochondria, purified from contaminating nerve-ending particles and myelin by passage through 1.2M-sucrose; (*b*) osmotically disrupted mitochondria, prepared by dilution of the mitochondria (originally suspended in 0.32M-sucrose) with 4vol. of water followed by twofold freezing and thawing; (*c*) mitochondrial hexokinase, purified after extraction with KCl and Triton X-100 and removal of detergent by a combination of ammonium sulphate fractionation and DEAE-cellulose chromatography (Thompson & Bachelard, 1970).

Hexokinase activity measurements. These were done in duplicate at 37°C in 100mM-triethanolamine–HCl buffer, pH7.6, in a Unicam recording spectrophotometer as described previously (Bachelard & Goldfarb, 1969). Mg^{2+} was maintained in excess at 6.75mM in order to ensure full binding of Mg^{2+} to ATP, which did not exceed 1mM. All determinations were performed under conditions of limiting concentrations of both substrates (glucose and $MgATP^{2-}$) (Cleland, 1963*b*; Clark, 1970). Kinetic results were analysed by regression lines where relevant, calculated by the method of least squares. Secondary plots were drawn from the intercepts of the primary kinetic plots according to Florini & Vestling (1957).

Reagents. The sodium salts of ATP, AMP and $NADP^+$, and glucose 6-phosphate dehydrogenase (EC 1.1.1.49, from yeast) were obtained from the Boehringer Corporation (London) Ltd., London W.5, U.K. The sodium salts of CTP, UTP, ITP, GTP, deoxy-ATP, 3′:5′-cyclic AMP and 2-deoxy-D-glucose were purchased from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. $\beta\gamma$-Methylene-ATP (free acid) was the product of Miles–Seravac (Pty.) Ltd., Maidenhead, Berks., U.K. *N*-Acetylglucosamine and all other chemicals were of the highest purity available from BDH Chemicals Ltd., Poole, Dorset, U.K.

RESULTS

Substrate kinetic analysis

Cytoplasmic hexokinase. The purified enzyme was chosen for this study since the presence of multiple forms (isoenzymes) might invalidate derivation of quantitative kinetic data. The experimental approach conformed to the general initial rate equation of Alberty (1953) and the constants quoted in the equation below follow his definitions:

$$v = \frac{V}{1 + K_A/[A] + K_G/[G] + K_{AG}/[AG]}$$

where [A] refers to $MgATP^{2-}$ and [G] to glucose.

The terms K_{AG}/K_G and K_{AG}/K_A were derived from the reciprocal substrate concentration at the common intersection point in double-reciprocal plots ($1/v$ versus $1/s$) (Dalziel, 1957; Florini & Vestling, 1957) or more directly from the substrate concentration of the intersection point in a plot of s/v versus s. The Michaelis constants, K_A and K_G, were derived from the secondary plots of the vertical intercepts, obtained from double-reciprocal plots, versus the reciprocal of the concentration of the second substrate (Florini & Vestling, 1957; Cleland, 1963*a*).

Double-reciprocal plots made with respect to glucose and $MgATP^{2-}$ are presented in Figs. 1*a* and 1*b* respectively; the corresponding secondary plots are Figs. 1*c* and 1*d* respectively. Values for K_{AG}/K_A were 0.058mM (Fig. 1*a*) and for K_{AG}/K_G, 0.40mM (Fig. 1*b*). The value for K_A was 0.35mM (Fig. 1*c*) and for K_G, 0.060mM (Fig. 1*d*). The lines of the plots of Figs. 1*a* and 1*b* converged with intersects near the abscissa. The alternate reciprocal plots

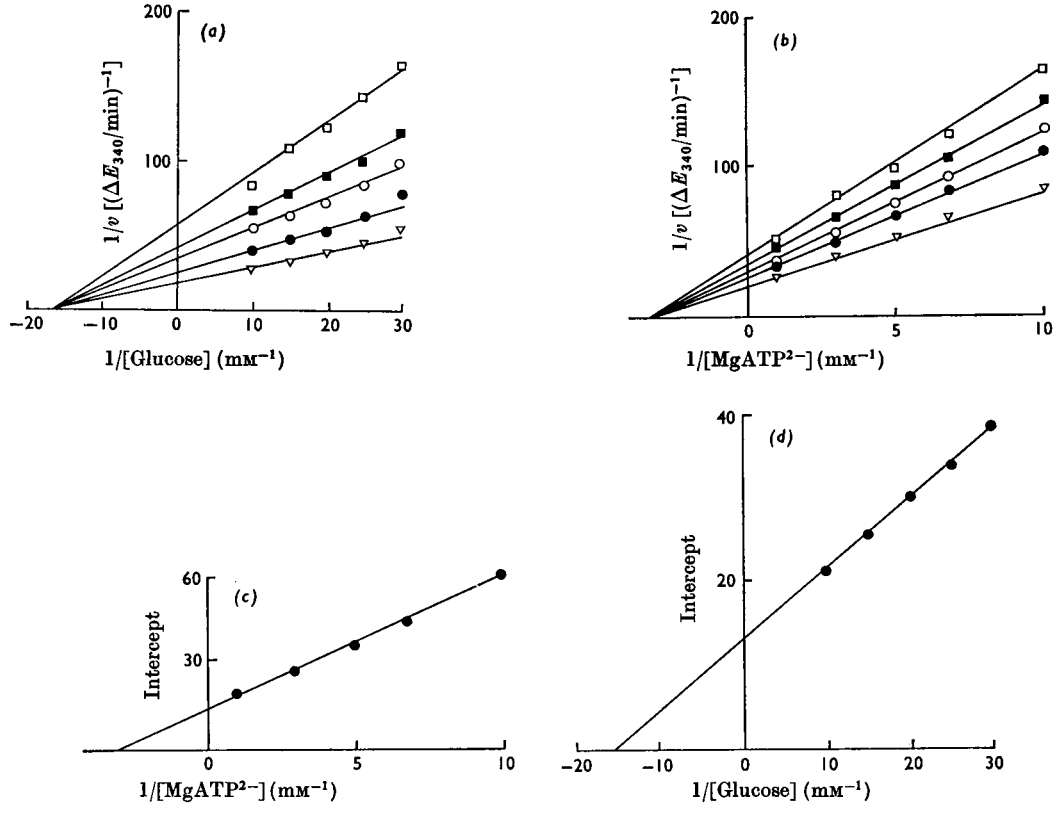

Fig. 1. Double-reciprocal plots ($1/v$ versus $1/s$) for cerebral cytoplasmic hexokinase. (*a*) Plot of $1/v$ versus 1/[glucose] with $MgATP^{2-}$ concentrations: □, 0.1mM; ■, 0.15mM; ○, 0.2mM; ●, 0.33mM; ▽, 1.0mM. (*b*) Plot of $1/v$ versus 1/[$MgATP^{2-}$] with glucose concentrations: □, 0.033mM; ■, 0.04mM; ○, 0.05mM; ●, 0.067mM; ▽, 0.10mM. (*c*) Secondary plot of intercepts from (*a*) versus 1/[$MgATP^{2-}$]. (*d*) Secondary plot of intercepts from (*b*) versus 1/[glucose]. Experimental details are described in the Materials and Methods section.

(s/v versus s) gave identical values for the constants. The intersection of the lines on the abscissa eliminates the possibility of a Ping Pong mechanism, as does the derivation of finite values for the terms containing K_{AG}: a Ping Pong mechanism requires that $K_{AG} = 0$. The intersection of the plots on the abscissa further implies that $K_A \cdot K_G = K_{AG}$ (Alberty, 1953). This relationship is verified by the experimentally determined values for these constants (Table 1).

Mitochondrial hexokinase. The kinetic properties of the three types of mitochondrial preparation (intact mitochondria, disrupted mitochondria and the purified, solubilized enzyme) were examined in view of the possibility of extraction resulting in altered kinetic properties. The enzyme exists in essentially one form in these preparations (Thompson & Bachelard, 1970) so that any complications due to multiple forms are not encountered. No difference between the three mitochondrial preparations was observed in the kinetic constants derived from primary or secondary plots (Table 2).

In contrast with the results for the cytoplasmic enzyme (Table 1) the kinetic constants for the mitochondrial hexokinase were not equivalent (Table 2). Thus K_G was not equal to K_{AG}/K_A and K_A was not equal to K_G/K_{AG}. However the ratios are constant, i.e.:

$$\frac{K_G}{K_{AG}/K_A} = \frac{K_A}{K_{AG}/K_G}$$

$K_A \cdot K_G = 0.075 \times 0.58 = 0.0435$; $K_{AG} = 0.010$; $K_A \cdot K_G$ is therefore approximately $4 \times K_{AG}$ and the non-equality is consistent with the non-axial intersection points observed. The cytoplasmic enzyme gave $K_A \cdot K_G$ equal to K_{AG} and axial intersection points.

Deoxyglucose as substrate. 2-Deoxy-D-glucose is known to be phosphorylated by brain hexokinase.

Table 1. *Substrate kinetic constants of cerebral cytoplasmic hexokinase*

The kinetic constants are defined in the text.

	Kinetic constants (mM)					
	Experimentally derived				Calculated	
Preparation	K_G	K_A	K_{AG}/K_A	K_{AG}/K_G	K_{AG} $[K_G(K_{AG}/K_G)]$	K_{AG} $[K_A(K_{AG}/K_A)]$
1	0.06	0.35	0.06	0.40	0.024	0.021
2	0.06	0.45	0.05	0.45	0.027	0.023

Table 2. *Substrate kinetic constants of cerebral mitochondrial hexokinase*

The kinetic constants are defined in the text. The sources of hexokinase are as described in the Materials and Methods section. Calculated K_{AG} values are as in Table 1.

	Kinetic constants (mM)					
	Experimentally derived				Calculated	
Source of hexokinase	K_G	K_A	K_{AG}/K_A	K_{AG}/K_G	K_{AG}	K_{AG}
Intact mitochondria (*a*)	0.08	0.62	0.018	0.14	0.011	0.011
Osmotically-disrupted mitochondria (*b*)	0.06	0.55	0.015	0.15	0.009	0.008
Purified mitochondrial enzyme (*c*)	0.05	0.55	0.018	0.13	0.009	0.007

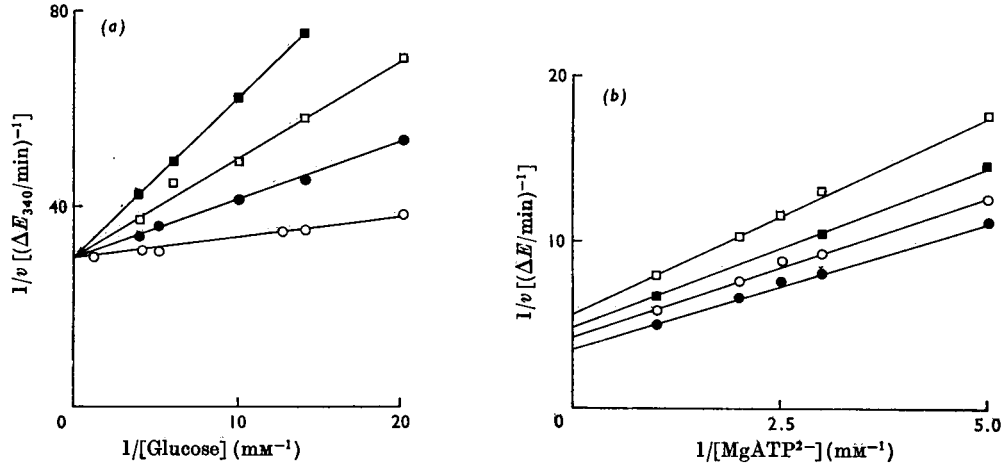

Fig. 2. Kinetic properties of inhibition of cerebral cytoplasmic hexokinase by 2-deoxyglucose. (*a*) Plot of $1/v$ versus 1/[glucose] at constant 1.0 mM-$MgATP^{2-}$ and deoxyglucose concentrations of: ■, 0.85 mM; □, 0.50 mM; ●, 0.25 mM; ○, nil. (*b*) Plot of $1/v$ versus $1/[MgATP^{2-}]$ at constant 0.1 mM-glucose and deoxyglucose concentrations of: □, 7.5 mM; ■, 5.0 mM; ○, 2.5 mM; ●, nil. Experimental details are described in the Materials and Methods section.

Although it has been shown to have a lower affinity than glucose for the enzyme (Sols & Crane, 1954; Grossbard & Schimke, 1966) little appears to be known of the kinetics of deoxyglucose interaction with the enzyme. The results of Fig. 2*a* show that deoxyglucose inhibits the phosphorylation of glucose by competition with glucose. The double-reciprocal plots ($1/v$ versus $1/s$) of cytoplasmic hexokinase activity in the presence of varying concentrations of glucose as substrate and different concentrations of deoxyglucose as inhibitor intersect on the ordinate. The Dixon plot ($1/v$ versus [I]) where [I] is the concentration of deoxyglucose, gave a measure of the K_i for the inhibitor of 0.25 mM. These plots were derived from initial velocity measurements at constant limiting concen-

tration of $MgATP^{2-}$ (1.0mM). Maintenance of constant limiting concentration of glucose (0.1mM) and varying the $MgATP^{2-}$ concentration showed (Fig. 2*b*) inhibition by deoxyglucose to be non-competitive with regard to $MgATP^{2-}$. Deoxyglucose can therefore be concluded to inhibit brain hexokinase by competition with glucose but not with $MgATP^{2-}$.

Dead-end inhibitor kinetic analysis

Selection of dead-end inhibitors. Our aim was to find a dead-end inhibitor for each of the two substrate sites. A variety of substrate analogues was tested for action as an inhibitor and also as a substrate. Clearly, an inhibitor would not fit the required category if it also acted as a substrate (e.g. 2-deoxyglucose). For these reasons *N*-acetylglucosamine proved to be a suitable inhibitor and is not phosphorylated by the enzyme (Harper & Quastel, 1949; Sols & Crane, 1954). The selection of an analogue for ATP was not so simple. The results provided some interesting information on the specificity of brain hexokinase. Of the ribonucleotide triphosphates tested ($\beta\gamma$-methylene-ATP, 3′:5′-cyclic AMP, GTP, CTP, UTP and ITP) only ITP acted as a substrate and was less than one-tenth as efficient as ATP. Rat brain hexokinase showed similar specificity for the nucleotides as substrates (Grossbard & Schimke, 1966).

None of the above nucleotides gave inhibition sufficient for use in inhibition kinetic studies. The results for these ribonucleotides are similar to those reported for yeast hexokinase (Martinez, 1961), but different from the results on ox brain of Ning *et al.* (1969) who found some of the nucleotides to be inhibitory. Surprisingly, $\beta\gamma$-methylene-ATP proved to be only slightly inhibitory (less than 5% inhibition was obtained with 2.5mM at 0.1mM-glucose and 1mM-$MgATP^{2-}$).

The efficient participation of deoxy-ATP as a substrate (71% of the activity obtained with ATP) was unexpected. AMP was the only satisfactory inhibitor of those tested. At 0.1mM-glucose and 1mM-$MgATP^{2-}$, 2mM-AMP gave 27% inhibition and 4mM-AMP gave 36% inhibition. The involvement of Mg^{2+} with inhibitor is probably negligible, since the binding constant of MgAMP (about $70M^{-1}$) is so much lower than that of $MgATP^{2-}$ (about $55000M^{-1}$) (Long, 1961). A synthetic ATP analogue (adenosine 5′-phosphohypophosphate) has recently been reported to be an active competitive inhibitor of interaction of ATP with yeast hexokinase (Remy, Setondji, Dirheimer & Ebel, 1970).

N-Acetylglucosamine inhibition of brain hexokinases. For the mitochondrial hexokinase, the double-reciprocal plots of $1/v$ versus $1/s$ at different concentrations of *N*-acetylglucosamine (Fig. 3) show that *N*-acetylglucosamine inhibition is competitive with glucose but that it is non-competitive

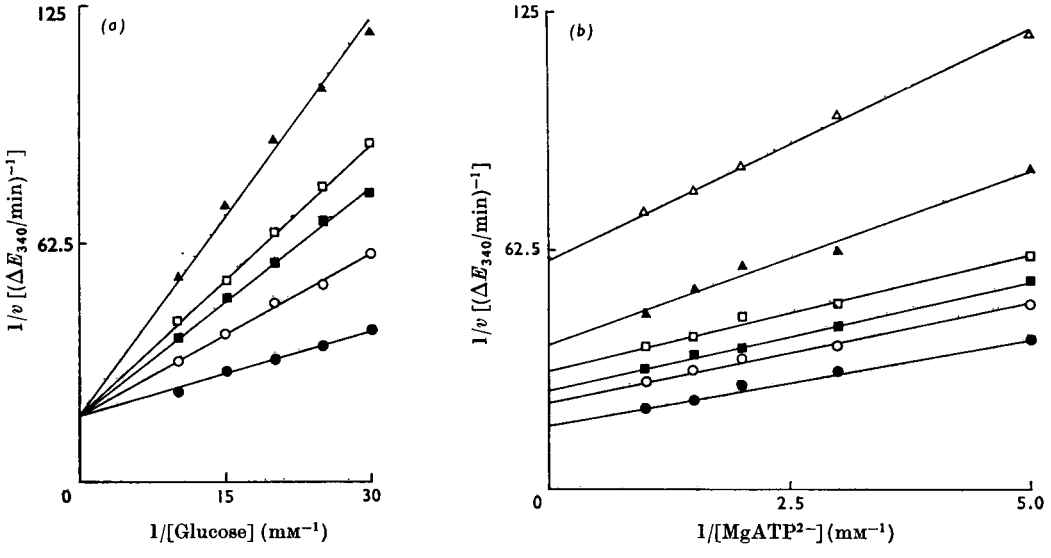

Fig. 3. *N*-Acetylglucosamine inhibition of cerebral mitochondrial hexokinase. (*a*) Plot of $1/v$ versus 1/[glucose] at a constant concentration of 0.5mM-$MgATP^{2-}$ and *N*-acetylglucosamine concentrations of: ▲, 1.0mM; □, 0.50mM; ■, 0.33mM; ○, 0.20mM; ●, nil. (*b*) Plot of $1/v$ versus 1/[$MgATP^{2-}$] at constant 0.10mM-glucose and *N*-acetylglucosamine concentrations of: △, 2.0mM; ▲, 1.0mM; □, 0.5mM; ■, 0.33mM; ○, 0.2mM; ●, nil. Experimental details are described in the Materials and Methods section.

Table 3. *Kinetic constants of N-acetylglucosamine inhibition of brain hexokinases*

K_i (inhibition constant) from the Dixon plot made with respect to competing substrate (glucose) or non-competing substrate ($MgATP^{2-}$). K_i (calc.) is derived from $i = -K_i(1+s/K_m)$ of the plots made with respect to the non-competing substrate. Secondary plots are the plots of the slopes (i) and intercepts (ii) of the primary plots ($1/v$ versus $1/s$) versus inhibitor concentration (Cleland, 1963*b*). The mitochondrial enzyme was the osmotically disrupted preparation (*b*) (see the Materials and Methods section).

Enzyme	Substrate varied	K_i from Dixon plots	K_i from secondary plots (i)	(ii)	K_i (calc.)
Cytoplasmic	Glucose	0.40	0.38	—*	
	$MgATP^{2-}$	1.6	1.6	1.55	0.43
Mitochondrial (*b*)	Glucose	0.32	0.30	—*	
	$MgATP^{2-}$	1.1	1.0	0.9	0.31

* Common intercept with competing substrate.

for $MgATP^{2-}$. Fully competitive kinetics were shown by intersection of plots at the ordinate when glucose was the varied substrate (Fig. 3*a*) but not when the $MgATP^{2-}$ was the varied substrate (Fig. 3*b*), where intersection occurred close to the abscissa. This was confirmed by the alternate plots of s/v versus s (not shown); parallel plots were observed when [glucose] was varied but not when [$MgATP^{2-}$] was varied. From Dixon plots ($1/v$ versus [*N*-acetylglucosamine]), when glucose was the substrate varied, the K_i (*N*-acetylglucosamine) was 0.32mM and when [$MgATP^{2-}$] was varied, the apparent K_i was 1.1mM. Calculation of the true K_i [$i = -K_i(1+s/K_m)$] gave a value of 0.31mM. Internal consistency of the kinetic measurements is confirmed by the similarity of kinetic constants obtained from the Dixon plots and from secondary plots of slopes and intercepts (Table 3).

Kinetic analysis of the *N*-acetylglucosamine inhibition of the cytoplasmic hexokinase gave similar results. Again the plots of $1/v$ versus $1/s$ provided clear evidence of competitive inhibition when glucose was the varied substrate but not when the $MgATP^{2-}$ concentration was varied. The K_i values for *N*-acetylglucosamine inhibition of the cytoplasmic activity from the Dixon plots were 0.4mM when [glucose] was varied and 1.6mM when [$MgATP^{2-}$] was varied. Calculation of the true K_i [$i = -K_i(1+s/K_m)$] gave a value of 0.43mM (Table 3).

Therefore, apart from slight quantitative differences in the plots of $1/v$ versus 1/[$MgATP^{2-}$] for the two enzymes and in the derived kinetic constants (Table 3), the kinetic properties of *N*-acetylglucosamine inhibition of the two brain hexokinases were identical. Fully competitive inhibition for glucose but not for $MgATP^{2-}$ occurred.

AMP inhibition of brain hexokinases. The mitochondrial enzyme was inhibited by AMP competitively when $MgATP^{2-}$ was the varied substrate. The plots of Fig. 4 ($1/v$ versus 1/[$MgATP^{2-}$]) provide clear evidence for competitive inhibition in the purified mitochondrial enzyme, and were the same for disrupted mitochondrial preparations. These results are in contrast with those of Ning *et al.* (1969) who found AMP inhibition not to be fully competitive for $MgATP^{2-}$ in the detergent-extracted particulate enzyme. In the present study, the competitive inhibition was also clear from plots of [$MgATP^{2-}$]/v versus [$MgATP^{2-}$] (not shown). The

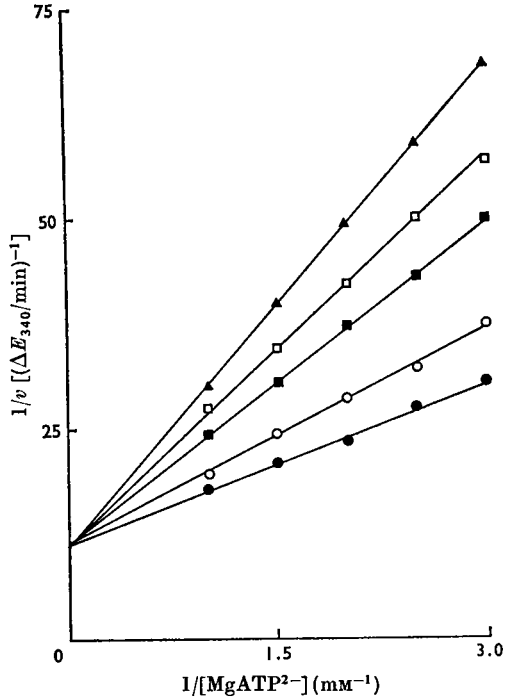

Fig. 4. AMP inhibition of purified cerebral mitochondrial hexokinase. Plot of $1/v$ versus 1/[$MgATP^{2-}$] at constant 0.1mM-glucose and AMP concentrations of: ▲, 8mM; □, 6mM; ■, 4mM; ○, 1mM; ●, nil. Experimental details are described in the Materials and Methods section.

Table 4. *Kinetic constants of AMP inhibition of brain hexokinases*

The mitochondrial preparations used were (*b*) osmotically disrupted mitochondria, (*c*) purified mitochondrial enzyme (see the Materials and Methods section). K_i values are defined in Table 3; in this case the competing substrate is $MgATP^{2-}$ and the non-competing substrate is glucose.

Enzyme	Substrate varied	K_i from Dixon plots	K_i from secondary plots (i)	(ii)
Cytoplasmic	$MgATP^{2-}$	1.9	2.0	—*
	Glucose	8.5	8.0	6.5
Mitochondrial (*b*)	$MgATP^{2-}$	2.5	2.5	—*
	Glucose	8.9	11.2	10.1
Mitochondrial (*c*)	$MgATP^{2-}$	4.4	4.3	—*
	Glucose	10.5	13.5	9.6

* Common intercept with competing substrate.

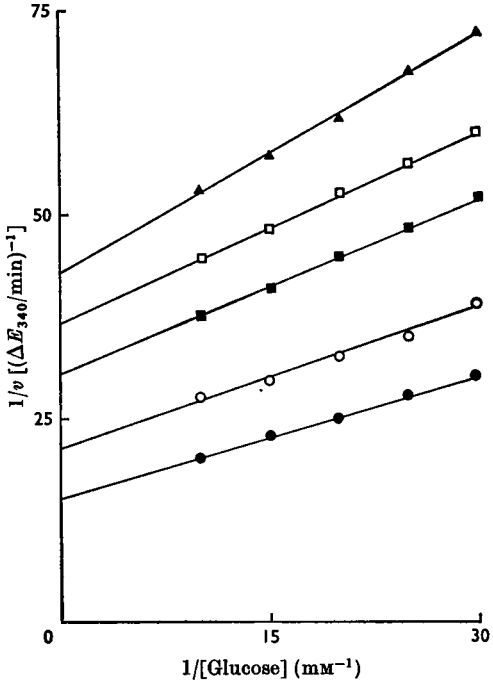

Fig. 5. AMP inhibition of purified cerebral cytoplasmic hexokinase. Plots of $1/v$ versus 1/[glucose] at constant 1 mM-$MgATP^{2-}$ and AMP concentrations of: ▲, 8 mM; □, 6 mM; ■, 4 mM; ○, 1 mM; ●, nil. Experimental details are described in the Materials and Methods section.

K_i for inhibition by AMP was 2.5 mM for the disrupted enzyme and 4.4 mM for the purified, solubilized mitochondrial enzyme, from the Dixon plots. Consistency of the constants was confirmed by comparison with the K_i values obtained from secondary plots (slopes versus [AMP]) of $1/v$ versus $1/[MgATP^{2-}]$ (Table 4). The cytoplasmic enzyme behaved qualitatively in identical fashion. The kinetic plots were similar to those of Fig. 4 and the K_i for AMP was 2.0 mM (Table 4).

When glucose was the substrate varied, all preparations showed similar non-competitive inhibition kinetics. The double-reciprocal plots converged just below the abscissa; the plots ($1/v$ versus 1/[glucose]) observed using the purified cytoplasmic activity are shown in Fig. 5. The plots of the results from the mitochondrial preparations were identical with those of Fig. 5. The non-competitive inhibition was also clear from plots of [glucose]/v versus [glucose], which converged near the abscissa. Values for the apparent K_i obtained from Dixon plots ($1/v$ versus [AMP]) were 8.5 mM for the cytoplasmic enzyme, 8.9 mM for the particulate enzyme and 10.5 mM for the mitochondrial activity after extraction and purification (Table 4). Cleland (1963*b*, p. 185) has emphasized the importance of the secondary plots for determination of K_i values, since intersection of primary plots on the abscissa in non-competitive inhibition may be fortuitous. The consistency of the results of Tables 3 and 4 gives support to the quantitative accuracy of the values derived. No qualitative differences were found between the particle-bound and the purified, solubilized-mitochondrial activities in substrate or inhibitor kinetic properties.

DISCUSSION

The results from kinetic analysis of substrate interaction with cerebral cytoplasmic hexokinase clearly eliminate the possibility of operation of a Ping Pong mechanism. The values for the kinetic constants of Table 1, where $K_G = K_{AG}/K_A$ and $K_A = K_{AG}/K_G$ are consistent with a random mechanism, in which no substrate–substrate interaction occurs, and where the presence of one substrate has no effect on the complexing of the second substrate (Alberty, 1953), but the possibility of an ordered mechanism could not be excluded solely on this basis. The latter mechanism would appear to be less likely but could only be eliminated definitely by inhibition studies.

The studies on the mitochondrial activity suggest

that the substrate kinetic properties of the enzyme are similar in intact mitochondria to those in disrupted mitochondria or in the purified enzyme after extraction from the mitochondria. As was found for the cytoplasmic enzyme, the mitochondrial enzyme exhibited kinetic properties which are not consistent with a Ping Pong mechanism.

The kinetic constants derived from the experimental results observed for the mitochondrial enzyme were not identical with those for the cytoplasmic activity. The values of Table 2, where K_G was not equal to K_{AG}/K_A and K_A was not equal to K_{AG}/K_G, emphasize the importance of secondary plots in deriving Michaelis constants stressed by Cleland (1963*b*). Internal consistency of the results is shown by experimental verification of the equality of the ratios, i.e.

$$\frac{K_G}{K_{AG}/K_A} = \frac{K_A}{K_{AG}/K_G} = 4.35$$

The values for the kinetic constants show a pattern quantitatively different from that of the cytoplasmic enzyme but also do not distinguish between random or an ordered type of reaction mechanism.

Random, rapid equilibrium enzymic mechanisms may be distinguished from ordered mechanisms by the use of fully competitive dead-end inhibitors in initial velocity studies. In the bimolecular case, both types of mechanism will conform to the equation below in the absence of inhibitors (Clark, 1970).

$$v = \frac{V}{1 + K_G/[G] + K_A/[A] + K_{iG} \cdot K_A/[GA]} \quad (1)$$

where [G] refers to glucose and [A] to $MgATP^{2-}$.

If the reaction mechanism is ordered with substrate G adding to the enzyme before substrate A, the initial velocity in the presence of a dead-end inhibitor, fully competitive with substrate G, is given by an equation of the type (2) below:

$$v_i = \frac{V}{1 + K_G(1 + [I]/K_i)/[G] + K_A/[A] + K_{iG} \cdot K_A(1 + [I]/K_i)/[GA]} \quad (2)$$

In such a case, double-reciprocal plots made with respect to substrate G will have a common intersection point on the ordinate. Double-reciprocal plots made with respect to substrate A will intersect at a point either on or above the abscissa.

If the added inhibitor competes with substrate A, the equation for the initial velocity is as (3) below:

$$v_i = \frac{V}{1 + K_G/[G] + K_A(1 + [I]/K_i)/[A] + K_{iG} \cdot K_A/[GA]} \quad (3)$$

In this case, double-reciprocal plots made with respect to substrate A will be fully competitive in character. Plots made with respect to substrate G will be characterized by a gradient that is independent of the inhibitor concentration. Thus a series of parallel straight lines will be obtained, the plot being uncompetitive in character.

In contrast, with a random rapid equilibrium mechanism, fully competitive inhibition with respect to either substrate will produce similar plotting patterns. Double-reciprocal plots made with respect to the competing substrate will be competitive in character, whereas those made with respect to the non-competing substrate will possess a common intersection point above, below or on the abscissa, if K_i (slope) is smaller than, greater than or equal to K_i (intercept), depending on the degree of substrate–substrate and inhibitor–substrate interaction (Cleland, 1963*b*; Clark, 1970). The initial-velocity equations will then be:

Competition with respect to G:

$$v_i = \frac{V}{1 + K_G(1 + [I]/K_i)/[G] + K_A/[A] + K_{iG} \cdot K_A(1 + [I]/K_i)/[GA]} \quad (4)$$

Competition with respect to A:

$$v_i = \frac{V}{1 + K_G/[G] + K_A(1 + [I]/K_i)/[A] + K_{iG} \cdot K_A(1 + [I]/K_i)/[GA]} \quad (5)$$

The pattern of inhibition of brain hexokinases by *N*-acetylglucosamine and by AMP is described by equations 4 and 5 and is therefore consistent only with a random type of reaction mechanism. *N*-Acetylglucosamine inhibited competitively with respect to glucose but not with respect to $MgATP^{2-}$; the inhibition when $MgATP^{2-}$ was the varied substrate was non-competitive. The evidence for this is that the intersects of the double-reciprocal plots (Fig. 3*b*) were on or near the abscissa when the non-competing substrate, $MgATP^{2-}$, was varied. This pattern eliminates the possibility of an ordered

mechanism with $MgATP^{2-}$ adding first, because in that case the inhibition when [$MgATP^{2-}$] was varied would have been uncompetitive rather than non-competitive.

Similarly, AMP inhibited competitively with respect to $MgATP^{2-}$ but not with respect to glucose. The intersection points of the double-reciprocal plots when [glucose] was varied (Fig. 5) were also on or near the abscissa. Thus the inhibition by AMP with respect to glucose is non-competitive.

This therefore eliminates the possibility of an ordered mechanism with glucose adding first because in that case the inhibition when [glucose] was varied would not have been non-competitive. Elimination of a Ping Pong mechanism from the study of the substrate kinetic properties and of ordered mechanism with glucose or $MgATP^{2-}$ reacting first with the enzyme leaves a random type of mechanism as the likely alternative.

The studies reported here lead to the conclusion that the reaction mechanism of brain hexokinase is of random type and that the three preparations studied (mitochondrial, both in particulate form and after solubilization and purification, and cytoplasmic) are similar in this regard. This qualitative similarity of kinetic properties supports the chromatographic evidence of similarity between cytoplasmic and solubilized mitochondrial activities previously reported (Thompson & Bachelard, 1970).

The close identity of these enzymes which occur in different sites within the cell is relevant to speculations on the role of the particulate enzyme in regulation.

The cytoplasmic enzyme has sufficient phosphorylating capacity under normal conditions of concentrations of substrates and products likely to occur *in vivo* (Bachelard & Goldfarb, 1969). However, this capacity could well be exceeded during the increased glycolytic rates which occur when the tissue is electrically stimulated *in vivo* and *in vitro*. If, as has been suggested (Wilson, 1968), increased rates of glycolysis may be dependent upon release to the cytoplasm of the particulate enzyme, this seems more feasible if the enzymes have identical properties.

Deoxyglucose, which acts as substrate for cerebral hexokinase, inhibits the phosphorylation of glucose competitively. The product of deoxyglucose interaction, deoxyglucose 6-phosphate, does not inhibit the enzyme, nor does it appear to act as a substrate in the brain for glucose 6-phosphate dehydrogenase or glucose phosphate isomerase (Sols & Crane, 1954). The competitive inhibition is of interest in view of possible hypoglycaemic effects of the deoxy sugar (Tower, 1960). Any inhibition of glucose utilization in the brain is therefore likely to be due in part to this and also to competition for glucose transport to the brain (Bachelard, 1971).

We are grateful to Professor McIlwain for his interest and encouragement, to Miss J. Edwards for her capable technical assistance, to the Research Fund of the Bethlem Royal Hospital and Maudsley Hospital, and the U.S. Public Health Service (Grant no. NS 07918) for financial support. M.F.T. was the recipient of a Medical Research Council Studentship for training in research methods.

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