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Adenine Nucleotides and Magnesium Ions in Relation to Control of Mammalian Cerebral-Cortex Hexokinase

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1. The kinetics of inhibition of brain soluble cytoplasmic hexokinase by ADP were examined in relation to variations in the concentrations of Mg^{2+} and ATP. The type of inhibition observed was dependent on the Mg^{2+}/ATP ratio. 2. ADP at Mg^{2+}/ATP ratios 2:1 exhibited inhibition of the 'mixed' type; at Mg^{2+}/ATP ratios 1:1 the inhibition appeared to be competitive with regard to ATP. 3. Inhibition by free ATP was observed when the Mg^{2+}/ATP ratio was less than 1:1. The inhibition was also of the 'mixed' type with respect to $MgATP^{2-}$. 4. The inhibitions due to ADP and to free ATP were not additive. The results suggested that there may be up to four sites in the soluble enzyme: for glucose, glucose 6-phosphate, ADP and $MgATP^{2-}$. 5. The 'free' non-particulate intracellular Mg^{2+} concentration was measured and concluded to be about 1·5mM. 6. The concentrations *in vivo* of Mg^{2+} and ATP likely to be accessible to a cytoplasmic enzyme are suggested to be below those that yield maximum hexokinase rates *in vitro*. The enzymic rates were measured at relevant suboptimum concentrations of Mg^{2+} and ATP in the presence of ADP. Calculations that included non-competitive inhibition due to glucose 6-phosphate (56–65% at 0·25mM) resulted in net rates very similar to the measured rates for overall glycolysis. This system may therefore provide a basis for effective control of cerebral hexokinase.

The factors that operate in the control of glycolysis in the brain have been suggested to include glucose transport and two glycolytic enzyme steps, hexokinase (EC 2.7.1.1) and phosphofructokinase (EC 2.7.1.11) (Lowry & Passonneau, 1964; Lowry, Passonneau, Hasselberger & Schulz, 1964). Regulation likely to occur at the hexokinase step would involve inhibition by the products, glucose 6-phosphate (Weil-Malherbe & Bone, 1951; Crane & Sols, 1953, 1954; Fromm & Zewe, 1962) and ADP (Sols & Crane, 1954; Fromm & Zewe, 1962). Glucose 6-phosphate inhibition of particulate rat and calf brain hexokinase was shown to be non-competitive with regard to glucose (Weil-Malherbe & Bone, 1951; Crane & Sols, 1953), but was reported to be competitive with regard to ATP (Fromm & Zewe, 1962) in calf brain. More recently the inhibition of guinea-pig brain hexokinase by glucose 6-phosphate has been suggested to be non-competitive with ATP also (Newsholme, Rolleston & Taylor, 1968). However, as pointed out by Newsholme *et al.* (1968) the different conclusions may have been due to differences in the criteria applied: Fromm & Zewe (1962) varied the concentration of Mg^{2+} + ATP, Newsholme *et al.* (1968) varied that of ATP.

The mode of inhibition by ADP of the particulate enzyme has also been a subject of controversy. Sols & Crane (1954) concluded ADP inhibition to be competitive with regard to ATP, whereas Fromm & Zewe (1962) reported that ADP inhibition affected both $V_{max.}$ and the apparent K_m when the concentration of ATP was varied, i.e. that ADP inhibition was of the 'mixed' type.

Rates of glycolysis, although capable of stimulation to greatly increased values for very brief periods of time, *in vivo* are, for many species, normally about 20–30 μmoles of glucose consumed/hr./g. of tissue (McIlwain, 1966, pp. 62, 98). Hexokinase activities in cerebral cortex, estimated *in vitro* under conditions of maximum activation, are considerably higher than this. Total hexokinase activities measured in this way have been reported to be from 600 μmoles of glucose phosphorylated/hr./g. in mouse whole brain (Lowry & Passonneau, 1964) to over 1200 μmoles/hr./g. in rat or guinea-pig cerebral cortex (Bennett, Drori, Krech, Rosenzweig & Abraham, 1962; Bachelard, 1967). Thus the total hexokinase may be potentially capable of activities 30–60-fold greater than the overall rate of glycolysis. From measurements of initial rates of glycolytic flux in ischaemia, Lowry & Passonneau

(1964) calculated that the upper limits were equal to only about 1·5–3% of the maximum total hexokinase activity in mouse brain and that an inhibition of 97% or more would be necessary for initial rate control. These authors suggested that the maximum inhibition likely to be provided by the concentrations of glucose 6-phosphate *in vivo* would amount to less than 70%. Even if the added inhibition due to ADP were taken into consideration, the total inhibition available would be insufficient when based on total hexokinase rates estimated under optimum conditions. It has been argued (Bachelard, 1967) that the soluble cytoplasmic hexokinase activity, 20–40% of the total (Kerly & Leaback, 1957; Balázs & Lagnado, 1959; Bachelard, 1967), is more than adequate to support glycolysis in the brain.

In the present work the kinetics of ADP inhibition of the soluble cytoplasmic hexokinase of bovine cerebral cortex were examined in relation to variations in the concentrations of Mg^{2+} and ATP. An attempt was made to evaluate the involvement of these factors in the enzymic activity in terms of the concentrations of Mg^{2+}, ATP, ADP and glucose 6-phosphate likely to be accessible to a soluble cytoplasmic system. During this study it became obvious that more information on the content of intracellular 'free' or non-particulate-bound Mg^{2+} available to the soluble enzyme was required, since it seemed unlikely that all of the Mg^{2+} of the cell would be accessible; a high proportion is bound to cellular elements such as phospholipids (Folch, Lees & Sloane-Stanley, 1957) or proteins (McIlwain, 1966, p. 31).

MATERIALS AND METHODS

Preparation of hexokinase. Ox brain was placed in a plastic bag immediately after its removal from the animal at a slaughterhouse, frozen by immersion of the plastic bag in a mixture of methanol and solid CO_2 in a vacuum flask and transported to the laboratory in the frozen state. The cerebral cortex was scraped off the frozen brain and homogenized in 5 vol. of 0·32 M-sucrose in a Teflon-pestle homogenizer (type C, clearance 0·15–0·23 mm.; A. H. Thomas Co., Philadelphia, Pa., U.S.A.). Dispersion involved ten passages of the pestle, rotating at 1500 rev./min. during 30–40 sec. with intermediate periods of cooling in ice. The homogenate was centrifuged at 78000 $g_{av.}$ for 1 hr. in the no. 30 rotor of a Spinco model L2-65 ultracentrifuge. The supernatant fraction was removed by decantation and the residue washed by dispersion by homogenization by hand in the original volume of sucrose and recentrifugation. The two combined supernatant fractions contained hexokinase activity (2·5 μmoles of NADPH formed/min./g. of tissue at 37°) equivalent to 22% of the total present in the homogenate (11·4 μmoles/min./g.).

The extract was purified slightly by $(NH_4)_2SO_4$ fractionation. The fraction that was precipitated between 25% and 70% saturation was dissolved in 50 mM-triethanolamine–HCl buffer, pH 6·5, and dialysed against three changes of the same buffer at 0°. The preparation contained 85% of the activity originally present in the combined supernatant fraction; the specific activity was 0·15 μmole of NADPH formed/min./mg. of protein. It was stable for weeks at −20° and had negligible 6-phosphogluconate dehydrogenase activity when tested for NADPH formation in the presence of 5 mM-sodium 6-phosphogluconate.

Assay of hexokinase activity. Hexokinase activities were measured in duplicate at 37° in semi-micro cuvettes in a Unicam SP. 800 recording spectrophotometer equipped with a constant-temperature cell housing. The incubation medium contained (final volume 1 ml.): triethanolamine–HCl buffer, pH 7·6 (0·1 M), $NADP^+$ (0·25 mM), glucose (2 mM), glucose 6-phosphate dehydrogenase (0·07 international unit) and, unless indicated otherwise, $MgSO_4$ (10 mM) and ATP (5 mM). After equilibration of the reaction mixtures at 37°, the assays were started by the addition of the hexokinase preparation (usually 20–50 μl.) and the formation of NADPH was followed by measuring E_{340}. Initial rates were recorded for at least 10 min. No change in E_{340} was observed when the incubation mixture contained all constituents except ATP or while the mixture was equilibrating before the addition of hexokinase. Reference cells contained all constituents except glucose and ATP.

Assessment of the non-particulate intracellular Mg^{2+} *concentration.* Guinea pigs were stunned by a blow to the back of the neck and killed by exsanguination. The cerebral hemispheres were rapidly removed, freed from white matter and slices (0·3 mm. in thickness) were cut dry with a bow-cutter [method (ii) of Bachelard, Campbell & McIlwain (1962)]. Only top slices of the cortex (60–90 mg.) were used. 'Rinsed slices' were immediately weighed and placed in 0·32 M-sucrose (adjusted to pH 7 with KOH) at 0° for 3 min., conditions designed to rinse out the extracellular ions (C. D. Richards & R. Sercombe, unpublished work). After the rinsing period the adhering moisture was removed from the surfaces of the slices by rapid repeated contact on a glass surface. The slices were then placed in the relevant homogenizing medium for tissue dispersion. 'Unrinsed slices' were cut, weighed and placed directly in the relevant homogenizing medium. Tissue dispersion was effected by placing the slice in 2·5 ml. of medium at 0° in a Teflon-pestle homogenizer (type A, clearance 0·16–0·24 mm.; A. H. Thomas Co.), and grinding involved ten passages at 0° of the pestle rotating at 1500 rev./min. A sample of the homogenate was retained and 2 ml. was centrifuged for 1 hr. at 150000 $g_{av.}$ in the no. 50 rotor of a Spinco model L2-65 ultracentrifuge. The supernatant fraction (S) was removed. Further washing of the residue was carried out by resuspending the residue in the original volume of medium and recentrifugation. The supernatant fraction (W) was removed. Residues (P) were suspended in deionized water (final volume 2 ml.). For determinations of Mg^{2+} the fractions were diluted with deionized water (v/v); homogenate (H), 1 in 20; S, 1 in 5; W, 1 in 5; P, 1 in 10. The relevant media were all diluted similarly and tested for Mg^{2+}. In no case was Mg^{2+} detected in the controls.

The Mg^{2+} concentrations were determined (Alcock & MacIntyre, 1966) with a Unicam SP. 90 atomic absorption spectrophotometer coupled to an SP. 20 recorder. The tissue fractions were diluted so that the protein concentration was below 0·5 mg./ml. (Willis, 1961) and the Mg^{2+} was determined without further treatment. A standard curve for

$MgCl_2$ in deionized water was linear between 1 and 50 μM and was not affected by the inclusion of bovine serum albumin (0·5 mg./ml.). The use of internal $MgCl_2$ standards in the diluted media controls and in controls containing known amounts of homogenate and residue showed that there was no interference with the Mg^{2+} determination from any of these sources. The media used, in addition to the sucrose described above were: 'imidazole', 10 mM-imidazole in 0·32 M-sucrose, adjusted to pH 7 with HCl; 'EDTA', 10 mM-K_2EDTA in 0·32 M-sucrose, adjusted to pH 7 with KOH.

Calculations of $MgATP^{2-}$. Estimates of the stability constant for $MgATP^{2-}$ vary from 24500 M^{-1} (Naninga, 1961) to values approaching 80000 M^{-1} (O'Sullivan & Perrin, 1964). Calculations of the data presented here, based on a mean value of 73000 M^{-1} (obtained at pH 8 at 30°; O'Sullivan & Perrin, 1964) or on the value of 55000 M^{-1} (extrapolated to 37° at pH 7·9; Burton, 1959), made little difference to the results. The results as presented are based on the stability constant of 55000 M^{-1}.

Reagents. The sodium salts of ATP, ADP, glucose 6-phosphate and 6-phosphogluconate were from Sigma (London) Chemical Co. Ltd., London S.W.6. $NADP^+$ (sodium salt) and glucose 6-phosphate dehydrogenase (EC 1.1.1.49) (from yeast) were purchased from the Boehringer Corp. (London) Ltd., London W.5. All other chemicals were of the highest purity available from British Drug Houses Ltd., Poole, Dorset. Measurements of pH were made with a pH-meter (Radiometer, Copenhagen, Denmark) with a glass electrode (type G 2222C).

RESULTS

Preliminary studies showed that the maximum activity of the soluble hexokinase from ox cerebral cortex was dependent on the Mg^{2+}/ATP ratio (Table 1). At 10 mM-Mg^{2+} and 10 mM-ATP the activity was slightly but reproducibly lower than at 10 mM-Mg^{2+} and 5 mM-ATP. At 5 mM-Mg^{2+} the activity decreased as the ATP concentration was increased from 2·5 mM to 10 mM. At 5 mM-Mg^{2+} and 10 mM-ATP the activity was only 55% of that at 10 mM-Mg^{2+} and 5 mM-ATP. Kinetic studies of the inhibition by ADP were therefore initially performed at two Mg^{2+}/ATP ratios (2:1 and 1:1).

Table 1. *Hexokinase activity and variations in* Mg^{2+} *and ATP concentrations*

Soluble hexokinase was prepared and assayed as described in the Materials and Methods Section.

Concn. of Mg^{2+} (mM)	Concn. of ATP (mM)	Hexokinase activity (ΔE_{340}/min.)	Hexokinase activity (% of optimum)
10	5	0·049	(100)
10	10	0·047	96
5	2·5	0·046	94
5	5	0·045	92
5	7·5	0·034	70
5	10	0·027	55

Inhibition of hexokinase by ADP. The Mg^{2+}/ATP ratio was maintained at 2:1 and the activity assayed over a range of ATP concentrations at various concentrations of ADP. Double-reciprocal plots (Fig. 1) showed that the ADP affected the $V_{max.}$ as well as the apparent K_m for ATP, an inhibition of the 'mixed' type similar to that reported by Fromm & Zewe (1962) for brain particulate hexokinase. However, if the Mg^{2+}/ATP ratio was maintained at 1:1 in a parallel series (Fig. 2), the intercept of the double-reciprocal plot was very close to the ordinate, indicating an apparent inhibition of the competitive type. The results suggest that the type of inhibition by ADP observed may depend on the Mg^{2+}/ATP ratio. In the presence of Mg^{2+}/ATP ratios 2:1 the apparent K_m for ATP was $3·5 \times 10^{-4}$ M. With Mg^{2+}/ATP ratios 1:1 the K_m value was approximately doubled.

The apparent K_i for ADP was obtained (Figs. 3*a* and 3*b*) from Dixon (1953) plots. When the Mg^{2+}/ATP ratio was maintained at 2:1 the apparent K_i was 4×10^{-4} M; at Mg^{2+}/ATP ratios 1:1 the K_i value measured was considerably higher ($8·5 \times 10^{-4}$ M).

Inhibition of hexokinase by excess of ATP. The observations (Table 1) that hexokinase activities were lower at Mg^{2+}/ATP ratios 1:1 than at 2:1 and lower still at 1:2, and the differences noted in the kinetics of inhibition by ADP at different Mg^{2+}/ATP ratios, led to a closer examination of the kinetics of inhibition by ATP in excess of the Mg^{2+} concentration. Excess of ATP was estimated by

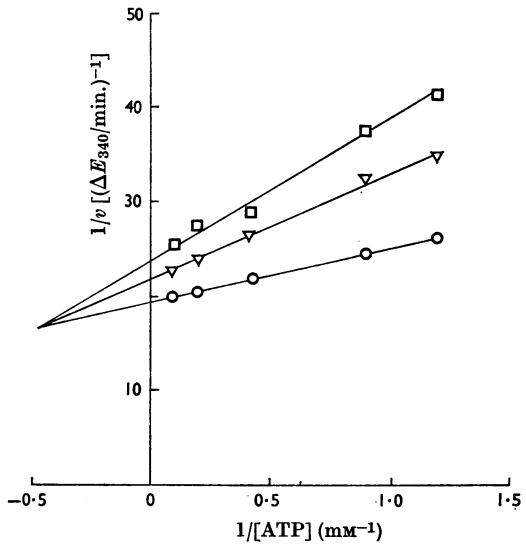

Fig. 1. Double-reciprocal plot of hexokinase activity versus ATP concentration when the Mg^{2+}/ATP ratio was maintained at 2:1. ○, No ADP; ▽, 0·7 mM-ADP; □, 1·4 mM-ADP.

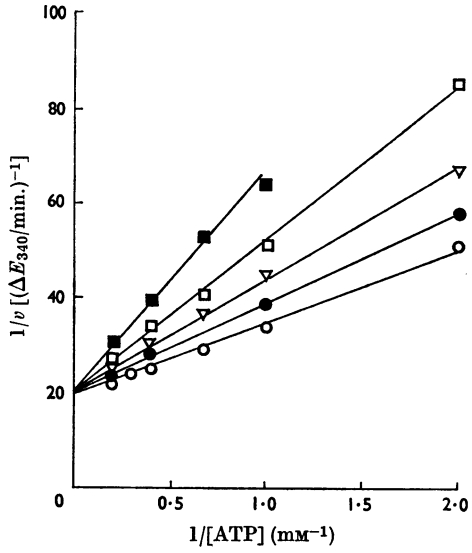

Fig. 2. Double-reciprocal plot of hexokinase activity versus ATP concentration when the Mg^{2+}/ATP ratio was maintained at 1:1. ○, No ADP; ●, 0·35mM-ADP; ▽, 0·70mM-ADP; □, 1·40mM-ADP; ■, 2·80mM-ADP.

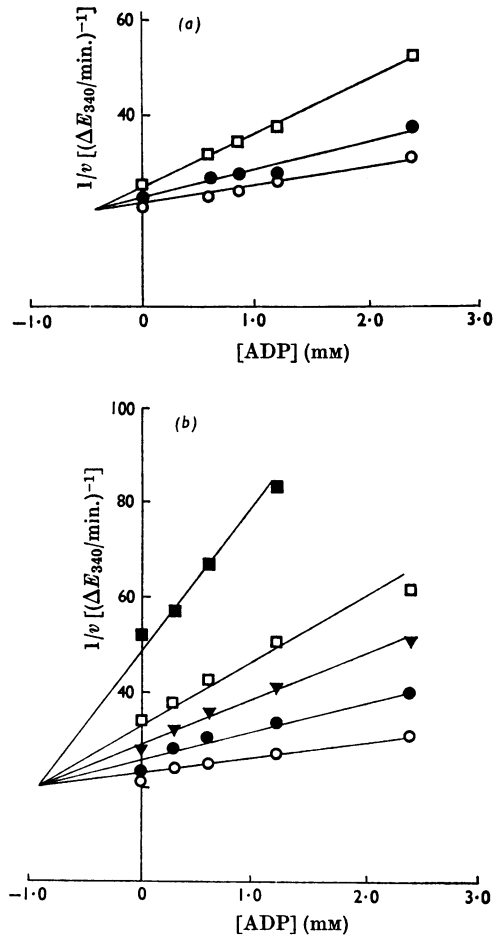

Fig. 3. Dixon plots of inhibition of hexokinase activity by ADP at (*a*) Mg^{2+}/ATP ratio 2:1 and (*b*) Mg^{2+}/ATP ratio 1:1. ○, 4·75mM-ATP; ●, 2·30mM-ATP; ▼, 1·40mM-ATP; □, 1·00mM-ATP; ■, 0·60mM-ATP.

subtracting the calculated amount of ATP bound to the Mg^{2+} present from the total ATP added. The double-reciprocal plots ($1/v$ versus $1/[MgATP^{2-}]$) obtained for various concentrations of ATP excess are shown in Fig. 4. The position of the intercept suggests that the excess of ATP affected both the $V_{max.}$ and the apparent K_m for $[MgATP^{2-}]$. Inhibition by free ATP would therefore also appear to be of the 'mixed' type, more complex than direct competition of the free ATP for an $MgATP^{2-}$ site on the enzyme. Dixon plots (Fig. 5) of the inhibition by excess of ATP gave an apparent K_i of 4×10^{-4}M, similar to the K_i obtained for ADP when the Mg^{2+}/ATP ratio was 2:1.

Inhibition of hexokinase by ADP and free ATP. The results from the kinetic studies of the inhibition by ADP and of the inhibition by excess of ATP raised the possibility of competition between ADP and free ATP as inhibitors. Accordingly the inhibition by ADP was compared at Mg^{2+}/ATP ratios 1:1 and 1:2 (Table 2). The results demonstrate clearly that the inhibition due to ADP and that due to free ATP are not additive. If the inhibition by ADP is taken as the criterion, the inhibition due to ATP decreases as the inhibition due to ADP increases. It is not possible from the data to assess which inhibition (i.e. that due to ADP or to free ATP) remains constant: neither may. However, it is clear that the total inhibition observed in the presence of both inhibitors is less than the sum of the inhibitions produced by each in the absence of the other.

Since the kinetics of inhibition by ADP appeared to be of the 'mixed' type with respect to ATP when the Mg^{2+}/ATP ratio was 2:1 (Fig. 1) it seems possible that the site for ADP may not be the same as that for $MgATP^{2-}$. Since the kinetics of inhibition by free ATP (excess) with respect to $MgATP^{2-}$ also appeared to be of the 'mixed' type (Fig. 4), and in view of the competition between inhibition by ADP and that by free ATP (Table 2) the possibility arises that the ATP may affect two sites: a site for ADP and a site for $MgATP^{2-}$.

Intracellular concentration of 'free' non-particulate Mg^{2+}. The proportion of total Mg^{2+} bound to particulate matter and unbound in the intracellular space was estimated by comparing the distribution in rinsed and unrinsed tissues after homogenization

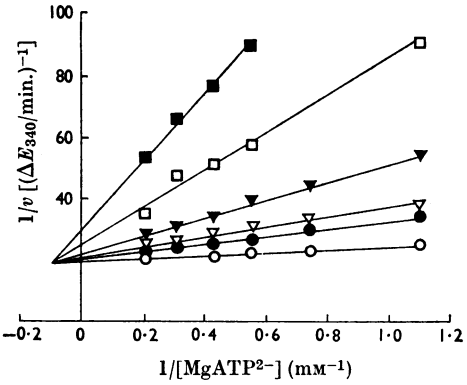

Fig. 4. Double-reciprocal plot of hexokinase activity versus $MgATP^{2-}$ concentration with excess of ATP. ○, Mg^{2+}/ATP ratio 2:1; ●, Mg^{2+}/ATP ratio 1:1; ▽, excess of ATP, 0·85mM; ▼, excess of ATP, 2·25mM; □, excess of ATP, 4·5mM; ■, excess of ATP, 7mM.

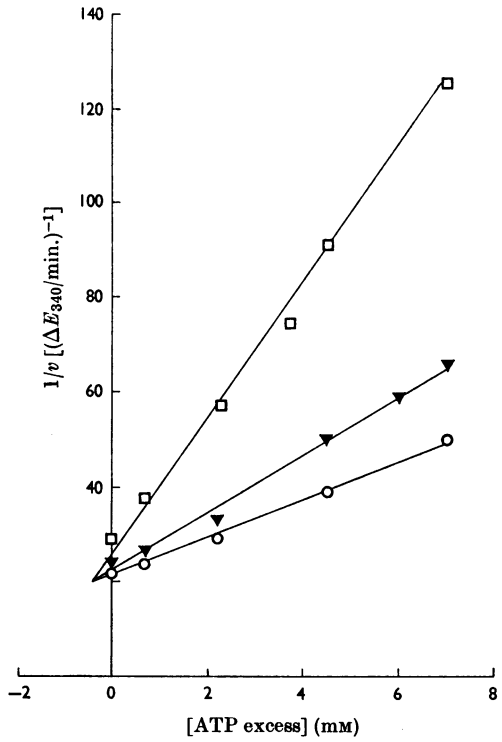

Fig. 5. Dixon plots of inhibition of hexokinase activity by excess of ATP. ○, 5·0mM-$MgATP^{2-}$; ▼, 3·5mM-$MgATP^{2-}$; □, 1·0mM-$MgATP^{2-}$.

in sucrose and centrifugation. The total Mg^{2+} content of unrinsed slices was $5·3 \pm 0·23$ μmoles/g. fresh wt., similar to the values previously reported for the cerebral cortex of other mammalian species

Table 2. *Inhibition of hexokinase by ADP and ATP*

Hexokinase activities were assayed at various concentrations of Mg^{2+}, ATP and ADP as described in the Materials and Methods section. Results are expressed as the percentage inhibition of the rate observed in the absence of ADP at Mg^{2+}/ATP ratios 1:1.

Concn. of Mg^{2+} (mM)	Concn. of ATP (mM)	Concn. of ADP (mM)	Inhibition (%)	Increased inhibition with excess of ATP (%)
5	5	0	0	—
		0·5	13·5	—
		1·0	21	—
5	10	0	40	40
		0·5	47	33·5
		1·0	45	24
2·5	2·5	0	0	—
		0·5	17·5	—
		1·0	27	—
2·5	5	0	32·5	32·5
		0·5	35	17·5
		1·0	37·5	10·5
1	1	0	0	—
		0·5	26	—
		1·0	31	—
1	2	0	13·5	13·5
		0·5	23·5	−2·5
		1·0	33	2

[4·7 μmoles/g. in human grey matter (Chang, Gover & Harrison, 1966); 6·3 μmoles/g. in rabbit cerebral cortex (Hanig & Aprison, 1967)]. The total Mg^{2+} content of the rinsed slices was $4·6 \pm 0·35$ μmoles/g., a difference significant to less than 1% ($P < 0·01$; Student's t test). The difference between the total Mg^{2+} contents of rinsed and unrinsed tissues, 0·7 μmole/g., represents an estimate of the 'extracellular Mg^{2+}', some of which may not be free in the extracellular space but may be loosely attached to particulate sites, for example on the outer surfaces of cell membranes. Of the cation present intracellularly in the rinsed slices, 0·7 μmole/g. was not bound to particulate matter and 3·6 μmoles/g. sedimented with the particulate matter. The non-particulate Mg^{2+} content of the unrinsed slices was 1·1 μmoles/g.

The net intracellular non-particulate Mg^{2+} was concluded to be 0·4 μmole/g. by subtraction of the 'extracellular' content (0·7 μmole/g.) from the total. If allowance is made for an 80% tissue water content (McIlwain, 1966, p. 27), the intracellular non-particulate-bound Mg^{2+} concentration would be approx. 0·5mM, considerably less than is usually assumed. However, these results suggested that some redistribution of Mg^{2+} may have occurred during the rinsing procedure as the Mg^{2+} contents of both the particulate and non-particulate fractions

Table 3. *Particulate-bound and non-bound* Mg^{2+} *in slices homogenized in various media*

Unrinsed slices were treated and the Mg^{2+} content was estimated as described in the Materials and Methods section. H, Homogenate; S, supernatant; W, washing; P, residue.

Medium	No. of preparations	Mg^{2+} content (μmoles/g.) H	S	W	P
Sucrose	4	5·5	1·05	0·8	3·45
Imidazole	2	5·7	1·9	0·8	2·7
EDTA	2	5·4	3·5	1·6	0·9

Table 4. *Calculated concentration of intracellular non-particulate* Mg^{2+}

Medium	Total non-particulate Mg^{2+} content* (μmoles/g.)	Net intracellular Mg^{2+} content† (μmoles/g.)	Concn. of Mg^{2+} in intracellular water‡ (mM)
Sucrose	1·1	0·4	0·5
Imidazole	1·9	1·2	1·5
EDTA	3·5	2·8	3·5

* From Table 3.

† After subtraction of 'extracellular' content (0·7 μmole/g.; see the text).

‡ By correction for tissue water content of 80% (McIlwain, 1966, p. 27).

decreased. It is possible that during tissue disruption also some redistribution had occurred. Some of the Mg^{2+} originally present in ionic form in the cellular water might have been redistributed by attachment to particulate matter and the result may therefore be misleadingly low. This implies a high affinity of the particulate matter for Mg^{2+}. If such is the case it is unlikely that the cation would be readily removed by a brief wash of the residue after centrifugation. This was tested (Table 3) and parallel experiments were performed in media containing reagents (imidazole and EDTA) that could be expected to form complexes with Mg^{2+}.

Unrinsed slices were used since the previous results indicated a possible redistribution of Mg^{2+} during rinsing. Brief washing of the sucrose residue released a further 0·8 μmole/g., suggesting that not all of the Mg^{2+} was tightly bound to particulate matter, and so it seems unlikely that a major redistribution had occurred during tissue disruption in sucrose. The use of 10mM-imidazole in sucrose resulted in the presence of a higher Mg^{2+} content (1·9 μmoles/g.) in the supernatant fraction and a further 0·8 μmole/g. was released by washing.

No stability constant appears to be available for the Mg^{2+}–imidazole complex, but by comparison with complexes with other cations for which K_i values have been recorded (Sillén & Martell, 1964) it would be considerably lower than the constant for the Mg^{2+}–EDTA complex (K_i $10^{8.7}$–$10^{9.7}$).

The Mg^{2+} content of the supernatant fraction after homogenization in 10mM-EDTA and 0·32M-sucrose was 3·5 μmoles/g. (Table 3). Washing with 5mM-EDTA has been shown to remove tightly bound Mg^{2+} from prewashed cerebral microsomal preparations (Goldfarb & Rodnight, 1969) and can be regarded as a strong competitor with particulate sites for Mg^{2+}. It follows that the use of EDTA results in an enhanced concentration of non-particulate Mg^{2+}, so that the actual concentration could be expected to be below the values observed in the presence of EDTA.

The intracellular concentration of 'free' or non-particulate-bound Mg^{2+} in the cerebral cortex was calculated (Table 4) to be 0·5mM (from the values obtained with sucrose) and 1·5mM (from the values obtained with 10mM-imidazole). The calculated concentration of 3·5mM from the use of EDTA can be regarded as above the true concentration, as discussed above.

It is difficult to assess with any precision the true Mg^{2+} concentration of the intracellular water of the tissue. The considerations discussed here serve to indicate that the concentration is likely to be well below 3·5mM and more than 0·5mM. The concentration observed with a reagent with a relatively mild affinity for Mg^{2+} (imidazole) was 1·5mM, which is intermediate between what may be taken as the extremes observed with sucrose or EDTA. The actual concentration of Mg^{2+} in the intracellular water of the cerebral cortex, although no precise value can be proposed, is certainly con-

Table 5. *Cytoplasmic hexokinase activities with added ADP at suboptimum concentrations of* Mg^{2+} *and ATP*

Hexokinase activities were assayed as described in the Materials and Methods section.

Concn. of Mg^{2+} (mM)	Concn. of ATP (mM)	Hexokinase activity (μmoles of NADPH/hr./g. of original tissue) No ADP	1·0mM-ADP
3·5	3·5	126	99
3·5	5·0	111	
2·5	2·5	122	89
2·5	3·5	105	
2·5	5·0	99	76
1·0	1·0	88	59
1·0	2·5	75	
1·0	5·0	52	
0·5	0·5	58	38
0·5	2·5	44	30

siderably lower than the concentrations often assumed in studies on Mg^{2+}-dependent enzymes. Until a better assessment can be made, the value obtained with imidazole (1·5mM) may be regarded as close to the actual concentration.

Hexokinase activities at suboptimum concentrations of Mg^{2+} and ATP in the presence of ADP. The rates of soluble hexokinase activity assayed with various concentrations of Mg^{2+} and ATP in the presence and absence of 1mM-ADP (the concentration *in vivo*; Estler & Ammon, 1967) appear in Table 5. The results are expressed as μmoles of NADPH formed/hr./g. of original tissue to facilitate subsequent discussion of the relevance to glycolytic rates *in vivo*, which are traditionally expressed in the form μmoles of substrate/hr./g.

DISCUSSION

The results from kinetic studies on ADP inhibition demonstrate the relevance of the Mg^{2+}/ATP ratio to the type of inhibition observed. In the presence of Mg^{2+}/ATP ratios 1:1 the inhibition by ADP appeared to be competitive with ATP; in the presence of Mg^{2+}/ATP ratios 2:1 the kinetic measurements were indicative of a mixed inhibition. This could account for different results obtained from previous studies. The importance of Mg^{2+}/ATP ratios has also been noted in studies on kinetics of inhibition by glucose 6-phosphate, where the interpretation of the kinetic data was different if the ATP concentration was varied (Newsholme *et al.* 1968) from that if the concentration of Mg^{2+} + ATP was varied (Fromm & Zewe, 1962). In the present study the results for ADP tend to confirm those of Fromm & Zewe (1962) in that inhibition by ADP appears to be 'mixed'.

Evidence is presented that inhibition by free ATP is also of the 'mixed' type. The K_i for inhibition by ATP (4×10^{-4}M) was similar to the K_i for inhibition by ADP; both were similar to the apparent K_m for ATP ($3{\cdot}5 \times 10^{-4}$M).

The non-additive inhibition by ADP and free ATP (Table 2) provides further evidence of a partial competition between ADP and ATP. This, taken together with the conclusion from the kinetics results, that inhibition by ADP and by free ATP are both of the 'mixed' type, leads to the possibility that the site for ADP and the site for $MgATP^{2-}$ may be different. ADP may interact at its own site and at the site for $MgATP^{2-}$, and ATP may also interact at the site for ADP and at the site for $MgATP^{2-}$. The possibility therefore exists of at least three sites on the enzyme: for glucose, $MgATP^{2-}$ and ADP. The suggestion that inhibition by glucose 6-phosphate is non-competitive with respect to glucose, ATP or ADP (Crane & Sols, 1953; Newsholme *et al.* 1968) indicates a fourth site. The inhibition by ADP is not likely to be due to competition with ATP^{-} for Mg^{2+}, as the binding constant for MgADP is only 5–6% of that for $MgATP^{2-}$ (Burton, 1959; O'Sullivan & Perrin, 1964).

Control of hexokinase. As there is likely to be little or no intracellular glucose in the tissue (Bachelard, 1967), it follows that phosphorylation of glucose occurs immediately on entry and therefore it seems reasonable to assume that the soluble hexokinase present in the cytoplasm, rather than the mitochondrial enzyme, is the one directly involved. An assessment of the actual rate of enzymic activity in its intracellular environment in terms not only of the concentrations of ADP and glucose 6-phosphate but also of the likely intracellular concentrations of Mg^{2+} and ATP would be considered more relevant than assessments based on the total activity of which the whole tissue is capable under maximally activating concentrations of Mg^{2+} and ATP.

The ATP concentration in rapidly frozen cerebral cortex has been reported to be 3–4μmoles/g. (Nyman & Whittaker, 1963; Estler & Ammon, 1967). The extramitochondrial intracellular concentration is at present difficult to assess. At least three factors operate to hinder any assessment: (i) ATP is depleted very rapidly during tissue handling unless rapid-freezing techniques are applied; (ii) the proportion of cerebral ATP stored intramitochondrially near its major site of formation to that in the cytoplasm is unknown; (iii) during tissue handling changes in the distribution as well as the concentration of ATP may occur. Accordingly, for the expression of relevant rates shown in Table 3, a range of ATP concentrations is included. Estimations of the concentrations of non-particulate-bound intracellular Mg^{2+} are not quite so difficult, since Mg^{2+} does not share the metabolic instability of ATP. However, the possibility that redistribution of Mg^{2+} might occur during tissue handling cannot be ignored. The intracellular concentration of 'free' non-particulate-bound Mg^{2+} was concluded to be about 1·5mM, from the measurements described with imidazole. The activities of soluble hexokinase at relevant concentrations of Mg^{2+} and ATP in the presence of 1mM-ADP (Table 5) were found to be from 38μmoles/hr./g. at 0·5mM-Mg^{2+} and 0·5mM-ATP to 89μmoles/hr./g. at 2·5mM-Mg^{2+} and 2·5mM-ATP. Inhibition by glucose 6-phosphate, shown to be non-competitive for the enzyme from the brains of various species (Weil-Malherbe & Bone, 1951; Crane & Sols, 1954; Sols & Crane, 1954; Newsholme *et al.* 1968), has also to be taken into account. No value appears to be available for the inhibition of the soluble hexokinase of ox brain by glucose 6-phosphate, although the particulate enzyme was

inhibited by 49% in the presence of 0·184mM-glucose 6-phosphate (Uyedo & Racker, 1965). Newsholme *et al.* (1968) reported inhibitions of over 80% of the soluble activity from guinea-pig cerebral cortex. This was measured in the present study by the method described by Uyedo & Racker (1965) with 0·25mM-glucose 6-phosphate (Lowry & Passonneau, 1964). With 0·1M-glucose the inhibition was 65% at 2·5mM-Mg^{2+} and 2·5mM-ATP and 56% at 0·5mM-Mg^{2+} and 0·5mM-ATP.

Inclusion of this inhibition would result in a net rate of 17μmoles/hr./g. at 0·5mM-Mg^{2+} and 0·5mM-ATP and 31μmoles/hr./g. at 2·5mM-Mg^{2+} and 2·5mM-ATP, values that are close to the rates observed for overall glycolysis (McIlwain, 1966, pp. 62, 98).

The argument is based on the assumption that it is the soluble enzyme that is relevant. If the calculations are based on the activity of the soluble enzyme in the presence of the likely intracellular concentrations of Mg^{2+}, adenine nucleotides and glucose 6-phosphate the requisite degree of control is approached.

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