Dissociation of Yeast Hexokinase under the Influence of Substrates†

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ABSTRACT: A study by sedimentation equilibrium ultracentrifugation was made of the pure isoenzymes, B, and C, of yeast hexokinase, which can each form a reversibly dissociating system under nondenaturing conditions. The apparent molecular weight (M_{app}) was determined, by computerized analysis, at points throughout the solution column in the cell, and hence related to varying protein concentration. By iterative fitting of curves based upon nonideal dissociation models, approximate values of the monomer–dimer association constants were estimated. The quantitative analysis showed that (at $I = 0.15$) there is no dissociation at pH 5.5, but that at pH 8 and above, dissociation is appreciable. Monomers of molecular weight 52,000 and dimers of 104,000 are the species involved. Dissociation is much augmented at higher ionic strengths. D-Glucose has a relatively small dissociating effect on native yeast hexokinase B, but this effect becomes large when a nucleotide substrate ADP (together with Mg^{2+}) is present with the glucose. Neither D-glucose 6-phosphate nor D-galactose can replace glucose in the latter effect. It is concluded that dissociation of the native dimer of this enzyme is an important element in the catalysis.

Hexokinase B is the most active of the hexokinase isoenzymes (A, B, and C) prepared in homogeneous form from baker's yeast (Lazarus *et al.*, 1966; Ramel *et al.*, 1971). It has been reported (Derechin *et al.*, 1966; Lazarus *et al.*, 1968) that hexokinase B, studied by sedimentation equilibrium at pH 5.5 by the method of Van Holde and Baldwin (1958), gave a value for the apparent M_z of 102,000. Similarly, for hexokinase P-II of Colowick and coworkers, which is equivalent to our hexokinase B, $M_w = 104{,}000$ was calculated from sedimentation coefficient determinations under comparable conditions (Schulze and Colowick, 1969). The isoenzyme C, as will be shown in the present report, cannot be distinguished in ultracentrifugation from hexokinase B.

Dissociation of hexokinase A or B was observed to occur at higher pH values (Derechin *et al.*, 1966; Lazarus *et al.*, 1968), and at pH 10.4 ($I = 0.10$) a monodisperse system was obtained with B, with M_z about 49,000, showing that at least a monomer–dimer system is involved. Concurrent results have been obtained by light-scattering measurements, in solutions up to pH 11, by Schulze and Colowick (1969). Recent electrophoretic analysis in our laboratory has shown that the hexokinase B molecule contains two identical subunits of M_w about 51,000 (Rustum *et al.*, 1971).

We report here more detailed studies upon the dissociation of the hexokinase B molecule as a function of the small molecules present. In particular, the special ability of substrates to dissociate the native molecule has been established. The occurrence of this latter phenomenon was suggested by the observation of Ramel and Schachman (Schachman, 1960; Ramel *et al.*, 1963), made using an earlier type of preparation of yeast hexokinase, that the presence of glucose led to a sharp decrease in its sedimentation coefficient. We demonstrate here the requirements for a substrate-induced dissociation of the pure isoenzyme, expressing the results in quantitative terms (with estimates of the dissociation constants for monomer–dimer equilibria). The analysis used displays point computations of the molecular weight values throughout the ultracentrifuge cell, and compares these to theoretical curves based upon a monomer–dimer equilibrium system (Derechin, 1971).

Materials and Methods

Hexokinases B and C were the completely pure preparations described by Ramel *et al.* (1971). The protein solutions were dialyzed continuously for 48 hr against the full medium to be used in the sedimentation. To obtain the ionic strengths stated, the calculated amounts of NaCl were added to the buffer solutions specified. For the dilute glycine buffers used, the fraction in the zwitterionic form was assumed to make no contribution to the ionic strength.

D-Galactose was Sigma "low glucose" grade. It was shown by gas–liquid chromatographic analysis (Bahl, 1969) to contain less that 1 part in 10,000 of any other hexose. ADP and glucose 6-phosphate were also from Sigma.

Sedimentation equilibrium experiments were performed at 3.5° in the Spinco Model E ultracentrifuge at low speed, using the reference frame method described elsewhere (Derechin, 1969). Solution columns were 3 mm long, centrifuged for 48–72 hr. The initial protein concentration, c_0, in each sample was first measured in a Hilger Model 154 interferometer. The relationship of c_0, and of the observed concentrations in the cell, to fringe numbers in the ultracentrifuge interferograms, was noted by Derechin (1971). A partial specific volume of hexokinase of 0.740 ml g^{-1} (calculated from amino acid composition data[1]) was assumed in all cases.

After equilibrium was reached, the interferograms were analyzed to give the values of ln c (where c is the protein concentration in g/100 ml at a given radial position, r, in the

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[1] E. A. Barnard, unpublished data.

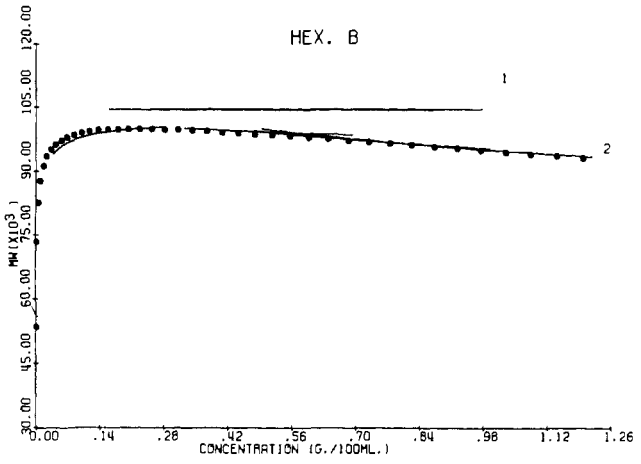

FIGURE 1: Computed (points) and observed (lines) molecular weights for hexokinase B: (1) in acetate–NaCl buffer (pH 5.5), $I = 0.15$; (2) in glycine–NaCl buffer, pH 8.0, $I = 0.10$. The computed points are based on the values of $K_2 = 500$ and $B = 7 \times 10^{-7}$. Each line represents an independent ultracentrifuge run: there are two in expt 1 and four in expt 2, illustrating the reproducibility attained over the whole range studied.

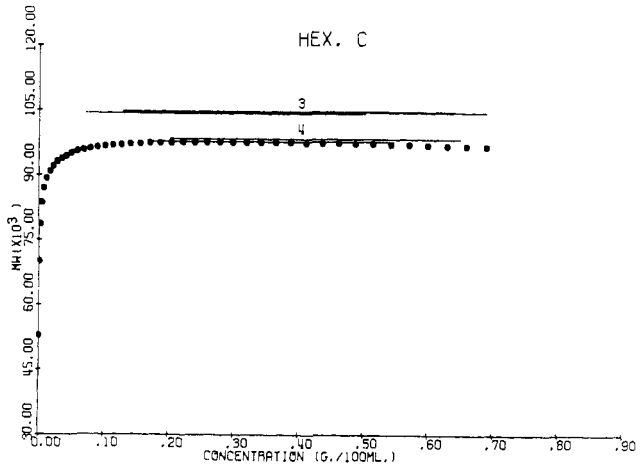

FIGURE 2: Computed and observed molecular weights of hexokinase C: (3) at pH 5.5 ($I = 0.15$) (three runs); (4) at pH 8.0 ($I = 0.10$) (two runs). The computed points are based on values of $K_2 = 450$ and $B = 7 \times 10^{-7}$.

cell). M_{app} was calculated[2] as a function of c, using the CDC 6400 computer and program WEIGHT, based upon the analytical methods described by Derechin (1971). For this purpose, the total concentration was calculated from assumed values of the mass equilibrium constants K_i ($i = 1, 2$), using

$$c = \sum c_i = \sum K_i c_1^i \quad (1)$$

$$M_w = \frac{\sum c_i M_i}{\sum c_i} \quad (2)$$

where $M_i = iM_1$. By successive approximation of the sets of values of M_{app} as a function of c, in comparison to the experimental values in the same range, the values of K_2 for

[2] Abbreviations used are: M_{app}, apparent weight-average molecular weight, as defined in eq 3 in the text; M_1, molecular weight of the monomeric (subunit) form.

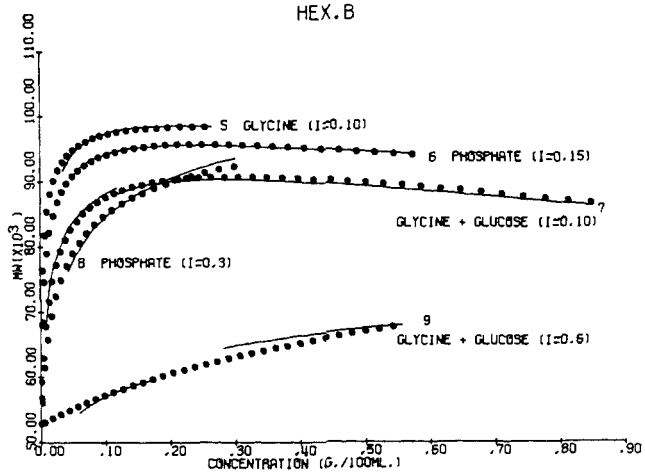

FIGURE 3: Computed and observed molecular weights of hexokinase B at pH 8.7 in: (5) glycine–NaCl, $I = 0.10$; (7) the same buffer containing 0.01 M glucose (two runs); (9) the same as 7 but with NaCl added to $I = 0.6$ (two runs); (6) sodium phosphate, $I = 0.15$; (8) sodium phosphate, $I = 0.30$.

a satisfactory fit were obtained (putting $K_1 = 1$). Here, M_{app} is defined according to Adams and Fujita (1963), as

$$\frac{1}{M_{app}} = \frac{1}{M_w} + Bc \quad (3)$$

where B is the second virial coefficient. The same iterative computational method yielded, also, a satisfactory value of B.

The readout data were plotted by a Calcomp Model 563 digital incremental plotter with an accuracy of one step (0.01 in., on either axis) per 120 ft paper length. All plots shown in this paper were made by the computer in this manner, without other adjustment. The continuous lines in all of the figures represent the experimental data, and the symbols represent the calculated theoretical points for a monomer–dimer equilibrium (Derechin, 1971).

Gel filtration was performed as described by Rustum *et al.* (1971), reading both the protein concentration and the hexokinase activity in the effluent, the two normalized values coinciding throughout in each case. Other materials and methods were as specified by Lazarus *et al.* (1968) or Rustum *et al.* (1971).

Results

Molecular Weight of Hexokinases. In sedimentation equilibrium at pH 5.5 ($I = 0.15$), hexokinase B showed no significant concentration dependence (Figure 1, curve 1). Nine experiments gave concurrent results, with mean M_w = 104,000 ± 1100 (std dev). The behavior suggested that the protein is homogeneous and undissociated, at least above 1-mg/ml concentration, if any possible nonideality is excluded. The same statement held true for hexokinase C (Figure 2, curve 3), which in four experiments yielded M_w = 104,800 ± 700.

Dissociation of Hexokinases. At pH 8.0 (at $I = 0.15$), hexokinase B displayed an average molecular weight (M_{app}) that varied with protein concentration across the cell, showing a flat maximum in M_{app} around a 2–5-mg/ml concentration (Figure 1, curve 2). The same type of behavior was also found at pH 8.7 in glycine or phosphate buffers (Figure 3,

TABLE I: Association Constants for Hexokinase Monomers in Various Conditions.[a]

Expt	pH	I	Buffer	Additions	K_2 (Mass)	K_2 (M)	BM_1	Monomer[b] (%)
2	8.0	0.10	Glycine		500	1.3×10^6	0.04	13
4	8.0	0.10	Glycine		450	1.1×10^6	0.04	14
5	8.7	0.10	Glycine		400	1.0×10^6	0.06	15
6	8.7	0.15	Phosphate		200	5.2×10^5	0.07	20
7	8.7	0.10	Glycine	Glucose	65	1.7×10^5	0.09	30
8	8.7	0.30	Phosphate		38	1.0×10^5	0.0	41
9	8.7	0.60	Glycine	NaCl + glucose	1	2.6×10^3	0.0	96
10	8.7	0.15	Glycine	MgADP + glucose 6-phosphate	400	1.0×10^6	0.05	15
11	8.7	0.15	Glycine	MgADP + galactose	40	1.0×10^5	0.03	40
12	8.7	0.15	Glycine	MgADP + glucose	3.5	9×10^3	0.03	80

[a] The experiment numbers correspond to those of the curves in the figures, with the concentrations of the added components as indicated there. The association constant was calculated both on a mass basis (for concentration units of g/100 ml) and a molar basis. [b] The percentage of the monomer (by weight) that will be present at a total protein concentration of 1 mg/ml. Note that all studies were on hexokinase B, except expt 4 on hexokinase C.

curves 5 and 6). The form of this concentration dependence denotes nonideal behavior in a dissociating system. Since in these experiments, and in all other dissociations of hexokinase under various conditions reported below, a value of M_{app} below 52,000 was not observed, the system was analyzed (as described under Methods, above) in terms of a monomer–dimer equilibrium, with a positive second virial coefficient, B (as defined in eq 3). This analysis provided a theoretical concentration dependence of M_{app} (shown by circles in the figures), based upon the value of K_2, the monomer–dimer association constant, and the value of B (positive or zero), that led to a good fit of these theoretical M_{app} points with the curve experimentally obtained.[3] This was true for all the curves discussed in this paper. It was seen that good agreement is obtained over the whole range of c investigated (up to 20 mg/ml in some cases) for a given value of K_2. The values obtained thus of the association constant of hexokinase B for various environmental conditions are shown in Table I. This table also shows the value of BM_1 required in each case.

The value of B needed was small, in the range 0–1.6×10^{-6}, for all of these cases. Moreover, the value of B does not affect the fit at the low concentration end, where ideality is approached, but does affect it greatly at the high concentration end. Nevertheless, a single value of B gave a reasonable fit throughout each curve, so that variations in K_2 were clearly discriminated (as can be seen from the values in Table I corresponding to the various curves).

Hexokinase C showed a dissociation behavior pattern identical with that of hexokinase B, when tested at pH 8.0 (Figure 2, curve 4; Table I).

Effect of Substrates on Hexokinase B Dissociation at pH 8.7. The effect of glucose on the dissociation of hexokinase B was examined at pH 8.7 (Figure 3, curve 7). It can be seen that the addition of glucose alone at 0.01 M somewhat favors dissociation, as shown quantitatively by about a sixfold change in K_2 (Table I).

Increase in ionic strength alone (using phosphate, without substrates present) significantly increased the dissociation of the dimer (Figure 3, curves 6 and 8). Increase in ionic strength (using NaCl), in the presence of (constant) glucose, again considerably increased the dissociation (Figure 3, curves 7 and 9).

The effect of a complete substrate mixture was examined by equilibrating hexokinase B with glucose (0.01 M), ADP, and $MgCl_2$ (each at 0.001 M, pH 8.7). In this case a large increase in dissociation was produced, over 100-fold (Figure 4, curves 12; Table I). In another experiment a true substrate mixture (0.01 M glucose–0.001 M ATP–0.001 M $MgCl_2$) was incubated at pH 8.7 (0.1 M glycine buffer, I = 0.15) with hexokinase B to equilibrium (30 min at 20°) and this mixture was dialyzed against the glycine buffer containing 0.01 M glucose 6-phosphate and 0.001 M ADP–0.001 M $MgCl_2$, followed by ultracentrifugation (Figure 4, curve 10). Very little dissociation was produced by this equilibrium mixture of products.

When the nonsubstrate, D-galactose, replaced D-glucose in the experiment with ADP and $MgCl_2$ present, the dissociating effect was much weaker than in the glucose–nucleotide medium (Table I). The effect of substrates in the phosphate medium was not studied, due to the competition for the Mg^{2+} by the phosphate ions.

The experiments in glycine media at pH 8.0 and 8.7, with and without the glucose–ADP–$MgCl_2$ mixture, were repeated with 0.001 M dithiothreitol present, in case dissociation is affected by the state of the thiol groups (Lazarus *et al.*, 1968) of this protein. In each case the same plot was obtained as in the absence of the reducing agent.

Gel Filtration Analyses. Further studies on the dissociation of hexokinase B were made by gel filtration on a column of Agarose in similar media (Table II). This method is convenient for studying the effects at low protein concentration, in this case in a narrow range around a mean of 0.4 mg/ml. A single, near-Gaussian peak was obtained in all the condi-

[3] When using eq 1–3, for a monomer–dimer system, it was first ascertained that simultaneous variation of M_1 to values <52,000 (in steps of 500) would lead to a worse fit of the experimental curves at *any* B and K_2 values. This is in accord with the maximum value of 104,000 actually observed for M_{app} (*i.e.*, M_2) under any conditions.

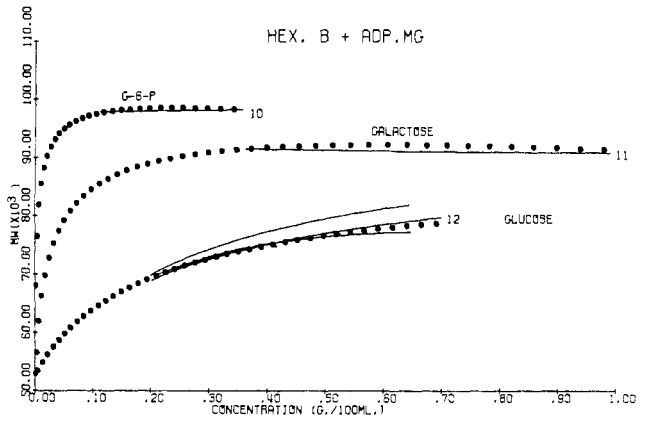

FIGURE 4: Computed and observed molecular weights of hexokinase B at pH 8.7 (glycine–NaCl, I = 0.15). Each medium contained 0.001 M ADP and 0.001 M $MgCl_2$, and the named sugar at 0.01 M concentration. In expt 12, three independent runs are shown.

tions listed in Table II, the position of the maximum of which was used for estimating M_{app} at 0.4 mg/ml. Dissociation in Tris buffer (I = 0.03) at pH 8.5 was slight, even at this low protein concentration. A high degree of dissociation was observed in 0.1 M phosphate, approximately as predicted from the M_{app} *vs.* c curve obtained by sedimentation measurements under nearly equivalent conditions (Figure 3).

The dissociating effect of glucose alone was just significant (Table II), but consistent with its effect (Figure 4, curve 7) measured by sedimentation in glycine medium. The combined effect of glucose and a nucleotide substrate (MgADP), in giving complete dissociation to half-molecules, was confirmed in this system. Used alone, neither MgADP nor the substrate D-fructose, had (at 10^{-2} M) more than a very minor dissociating effect. In the presence of the MgADP, however, D-fructose was as effective as D-glucose in dissociating hexokinase B in these conditions.

TABLE II: Gel Filtration of Hexokinase B in the Presence of Ligands.[a]

Reagent Added[b]	App Mol Wt[c]
None	90,000
0.1 M sodium phosphate	58,000
D-Glucose	85,000
ADP + Mg^{2+}	85,000
D-Glucose + ADP + Mg^{2+}	52,000
D-Fructose	85,000
D-Fructose + ADP + Mg^{2+}	50,000

[a] A column (45 × 2 cm) of Agarose A-0.5 m was equilibrated in the medium specified. The hexokinase (8 mg/ml) was dialyzed against the buffer concerned, and applied in a 0.5-ml volume. 2-ml fractions were collected; a single peak was present in every case. The observed molecular weight was calculated from a calibration plot (Rustum *et al.*, 1971). [b] The medium was 0.1 M Tris-chloride (pH 8.5) in all cases, except for the one experiment when 0.1 M sodium phosphate (pH 8.5) was used alone. Sugars, ADP, and $MgCl_2$ when present were at 0.01 M concentration. No difference was found in the result when ADP and $MgCl_2$ were used, instead, at 0.001 M concentration (as used in the ultracentrifugal experiments) in the experiment with 0.01 M glucose. [c] The value of M_w to which the elution volume corresponds. Since dissociation is involved, this value is apparent only.

Discussion

The data presented here show that hexokinases B and C each behaves (at pH 8–9) as a self-associating system. The upper and lower limits observed for the molecular weights indicate that a monomer–dimer equilibrium is involved. The molecular weight of the monomer is seen to be close to 52,000. This agrees with the value (51,000) determined for the subunits of these proteins by polyacrylamide gel electrophoresis in sodium dodecyl sulfate media (Rustum *et al.*, 1971).

The molecular weight of hexokinase B in its dimeric state is obtained independently, from the sedimentation results at pH 5.5, where no evidence for dissociation was found in the concentration range studied. From those results, M_w is in the range 104,000 ± 1000. These results for M_w are in excellent agreement with those obtained previously (Lazarus *et al.*, 1968) for hexokinases A and B at pH 5.5 in the same conditions, when M_z was determined by the sedimentation equilibrium method of Van Holde and Baldwin (1958). The coincidence of the M_w and M_z values is good evidence that a single species is involved at pH 5.5 in each case. The present results, taken with those of Rustum *et al.* (1971), show clearly that the equilibrium involved is between the native protein and its two subunits.

Most of the studies were pursued with hexokinase B, but hexokinase C also yielded the same M_w values. Hexokinase C is known to be a form convertible to hexokinase B without peptide-bond cleavage (Ramel *et al.*, 1971), so that identity of their M_w values in the dimer form, and again in the monomer form, is to be expected.

In disagreement with some of our results, Easterby and Rosemeyer (1969) have reported, using the sedimentation equilibrium method of Van Holde and Baldwin (1958), that the true molecular weight of yeast hexokinase A is 111,000 and that dissociation can occur to 28,000 molecular weight subunits. However, the method of preparation of their hexokinase A, and its freedom from protease contamination, are not described. The smallest M_{app} observed in the plots used to make these deductions was 40,000 (at I = 3.0, pH 7.0). The deduction of a value of M_w = 28,000 from these observed M_{app} values was made by those authors using the method of Sophianopoulos and Van Holde (1964) in which the relationship

$$M_z = (n + 1)M_1 - nM_1^2/M_w \qquad (4)$$

is employed. This relationship was deduced by the latter authors for the case of a two-species (monomer and n-mer) equilibrium. The postulated case of a three-species stepwise equilibrium would, in fact, require a much more complex treatment, as shown by Roark and Yphantis (1969). Further, the second dissociation step had to be assumed arbitrarily not to occur in the range where the first is observable. The M_{app} values observed at the high ionic strength by Easterby and Rosemeyer (1969) are attributable, in our view, either to a slight proteolysis in that medium, or to an artifactal effect on M_{app} of the high-salt medium, such as a preferential hydration (Aune and Timasheff, 1970).

A distinct dissociating effect of ionic strength was seen

(Figure 3, curves 6–9; Table I), both with sodium phosphate and sodium chloride additions. An effect of ionic strength, and of glucose, in dissociating hexokinases P-I and P-II (which are equivalent to A and B), has previously been indicated in measurements of apparent M_w (by light scattering) or $s_{20,w}$ by Schulze and Colowick (1969), at a single protein concentration. The present results are compatible with those, but ours were obtained in different media and at a higher pH, and the influence of ionic strength and protein concentration on the dissociation is expressed quantitatively here.

In the partly dissociated systems involved, it can be noted, from our M_{app} plots, that a measurement of molecular weight made at a single protein concentration will often provide a rather poor estimate of the relative degree of dissociation existing. For example, curves 6 and 8 in Figure 3 (both in phosphate media, at different I) would show only small differences in M_w measured at c = 2–3 mg/ml, while the actual K_2 values, calculated from data over a wide range of c, differ by a factor of 5. This illustrates the value of using point computations throughout the range of c that is represented across the cell, especially when (as is usual) nonideality is present.

In the early studies of yeast hexokinase, it was believed that 0.01 M glucose alone could completely dissociate the molecule (Ramel *et al.*, 1963). It is now known that the earlier method of preparation used gave a partially proteolyzed enzyme which had suffered chain cleavage and was easily dissociated (Lazarus *et al.*, 1966; Rustum *et al.*, 1971). In fact, with the intact hexokinase, glucose alone has a relatively small, although definite, dissociating effect (Table I, expt 5 and 7). This effect can be greatly augmented by high ionic strength (expt 9).

The effect of glucose (at low ionic strength) is entirely different when the nucleotide is present (expt 12). Since Mg ADP has virtually no additional effect in the absence of the glucose (Figure 4, curve 10; Table II), we conclude that a ternary complex, hexose–nucleotide–hexokinase, is formed as a distinct species by the monomeric form of the enzyme.[4]

The lack of effect of the true equilibrium substrate mixture can be ascribed to a poor binding of glucose 6-phosphate to the enzyme. At pH 8 and above, the equilibrium constant is such (Robbins and Boyer, 1957) that virtually no free glucose will be present. With other preparations of this enzyme, glucose 6-phosphate has been shown to be a very poor competitive inhibitor (Noat *et al.*, 1968; Fromm, 1969), a finding confirmed[5] on hexokinase B, and the K_m value for glucose 6-phosphate in the catalytic reaction with excess MgADP has been reported (at pH 7.6, 30°) as 0.08 M, about 600 times greater than that for glucose under equivalent conditions (Gamble and Najjar, 1955; Noat *et al.*, 1969). In conditions comparable to ours (Kosow and Rose, 1970), MgADP has a fairly strong affinity for yeast hexokinase B in the presence of saturating glucose. We presume, therefore, that the binding of glucose and MgADP together on the enzyme gives rise to the same type of interaction as in the forward reaction of the substrates. The evidence obtained indicates that dissociation by the substrates, in the forward direction of the reaction, is an important element in the mechanism of action of yeast hexokinase.

[4] While it has been reported (Noat *et al.*, 1969) that one molecule of glucose is bound by each molecule of yeast hexokinase in the absence of nucleotides (at pH 8.5 in glycylglycine buffer), the enzyme employed had M_w = 49,000 even in the absence of glucose and of phosphate (at I = 0.05, pH 7.5 or 8.5, c = 1 mg/ml), so that a different (presumably proteolyzed) form of the protein was involved.

[5] M. Tunis and E. A. Barnard, manuscript in preparation.

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