A Glucokinase from *Saccharomyces cerevisiae*

P. K. MAITRA

From the Molecular Biology Unit, Tata Institute of Fundamental Research, Bombay 5, India

SUMMARY

An ATP:D-glucose 6-phosphotransferase (EC 2.7.1.2) or glucokinase from *Saccharomyces cerevisiae* is described. The enzyme has been purified about 100-fold from the crude extracts of a hexokinaseless mutant derived from a haploid strain of *S. cerevisiae*. The K_m values for glucose and ATP are 28 and 50 μM, respectively. The maximal velocity of this enzyme toward fructose is 0.4% of that of glucose. The enzyme showed a marked heterogeneity when sedimented in a sucrose density gradient, its molecular weight ranging from 144,000 to a value in excess of 200,000. Yeast glucokinase shares a number of properties with yeast hexokinase (EC 2.7.1.1) in the semiconstitutive nature of its synthesis with glucose, its broad pH optima, and inhibition with ADP and N-acetylglucosamine. Its mode of reaction with glucose and ATP, as indicated by initial velocity patterns, appears to be random.

Although specific glucokinases (EC 2.7.1.2) have been described in a number of bacteria (1, 2) and mammals (3, 4), such an enzyme has not been described in yeast. Recently, Rustum and Ramel have noticed a glucose-specific enzyme in baker's yeast (5). While studying the properties of 2-deoxyglucose-resistant mutants of *Saccharomyces cerevisiae* we found that resistance to this sugar analogue does not necessarily confer glucose negativity. On the other hand, some of these mutants are unable to grow on fructose. The wild type, however, grows equally well on glucose or fructose. This was soon traced to the presence of an enzyme that phosphorylates glucose but not fructose. We describe here the partial purification and the properties of this enzyme from *S. cerevisiae*.

EXPERIMENTAL PROCEDURE

Strains and Media—A wild type haploid strain of *S. cerevisiae* of mating type *a* obtained from Dr. S. N. Kakar of the Department of Genetics, Punjab Agricultural University, was used in these studies. Unless stated otherwise cells were grown aerobically on a rotary shaker at 30° in a medium containing peptone and yeast extract in the proportion of 1.0 and 0.3 g/100 ml; extra carbon sources, such as glucose (100 mM), galactose (100 mM), fructose (100 mM), or acetate (40 mM), were added whenever indicated. A salt-vitamin medium of the following composition was used in some experiments: $(NH_4)_2SO_4$, 38 mM; potassium

phosphate (pH 7.4), 20 mM; NaCl, 8 mM; $MgSO_4$, 3 mM; $FeSO_4$, 18 μM; $ZnSO_4$, 3.5 μM; $CuSO_4$, 0.4 μM; inositol, 55 μM; pyridoxine, 10 μM; thiamine, 6 μM; pantothenic acid, 4 μM; biotin, 0.02 μM; yeast extract, 0.03 g/100 ml; carbon sources as above.

Mutagenesis—Cells grown overnight in salt-vitamin-galactose medium were treated in the exponential phase with 0.1 mg of N-methyl-N'-nitro-N-nitrosoguanidine per ml for 60 min. After washing, the mutagenized cells were grown overnight in a number of separate tubes in the galactose medium containing 1 mM 2-deoxyglucose (6). Washed cells from each tube were plated on this medium; the surviving clones were tested for hexokinase (+glucokinase) activity after growing in stationary culture in yeast extract-peptone-galactose medium. Colonies containing nearly 10% of the glucose-phosphorylating activity of the wild type were further purified on galactose-yeast extract-peptone medium containing up to 10 mM 2-deoxyglucose. Those which were characterized by a relatively rapid rate of growth were finally picked after selection from the salt-vitamin-galactose medium containing 50 mM 2-deoxyglucose. The clone finally selected was designated 7L1.

Enzyme Assay—In most experiments, the glucose-phosphorylating activity was estimated by measuring the rate of glucose-6-P formation. The reaction mixture consisted of 10 mM glucose, 1 mM ATP, 0.1 mM NADP+, and 0.3 unit of glucose-6-P dehydrogenase in a 50 mM triethanolamine buffer, pH 7.4, containing 10 mM $MgCl_2$. Fructose-phosphorylating activity was measured in the same system as for glucose except that it contained 10 mM fructose and 1 unit of crystalline P-glucose isomerase in place of glucose. The reaction was followed fluorometrically (7) in a 2-ml quartz cuvette at room temperature (22–24°) in an Eppendorf photometer whose photomultiplier was energized at 900 volts; the amplified output after voltage compensation was applied across a Varian G14 strip chart recorder operated at 10 mv. For most studies an RC filter of 0.5 sec time constant was interposed between the photometer and the recorder. A full scale deflection of 5 mμmoles of NADPH was easily obtained. In some experiments the sugar-phosphorylating activity was measured by monitoring the rate of ADP formation from ATP in a system containing 10 mM $MgCl_2$, 10 mM sugar, 1 mM ATP, 30 μM NADH, 1 mM P-enolpyruvate, 0.5 unit of pyruvate kinase, and 1 unit of lactic dehydrogenase in 50 mM triethanolamine hydrochloride neutralized to pH 7.4 with KOH. Since ATP contained significant amounts of ADP, reaction was started with the sugar solution. The equality of the rate of purified glucokinase measured by either glucose-6-P or ADP assay shows the stoichiometric relation between the two products. All enzyme units are expressed as the amount of enzyme catalyzing the con-

version of 1 μmole of substrate per min under the conditions described.

The enzyme solution used was either a 20,000 × *g* supernatant of a French pressure cell extract or a fraction derived from it or, in the screening studies, a suspension of toluenized cells. For toluenization a 2-ml suspension of yeast cells in 50 mM potassium phosphate (pH 7.4), 2 mM β-mercaptoethanol, and 2 mM EDTA was treated with 2 drops of toluene and kept shaking at 37° in a reciprocating shaker for 20 min.

The differential rate of glucokinase synthesis was determined by examining the glucose-phosphorylating activity of toluenized cell suspensions. Overnight culture of 7L1 from yeast extract-peptone-acetate medium was diluted in fresh medium containing added carbon sources. When the steady state of exponential growth was achieved, samples of 10 ml were centrifuged, resuspended, and toluenized. The mass of the culture was estimated by its extinction at 650 mμ in a 1-cm path.

Chemicals—All sugars were of D-configuration. Galactose for screening mutants contained 3% glucose and was freed from glucose by incubating the galactose solution with a washed 10% suspension of a glucose-grown culture of a hybrid yeast, *Saccharomyces fragilis* × *Saccharomyces dobzhanskii*, for 3 hours at 37°. The suspension was centrifuged, passed through membrane filters, and then sterilized by autoclaving. It contained less than 0.003% glucose. Sugars used as substrates for glucokinase were treated with glucose oxidase (Worthington) to remove any trace of glucose. Particular care was taken to ensure that fructose (Sigma) did not contain any glucose; absence of glucose in glucose oxidase-treated fructose was ensured by lack of NADPH formation in a glucose assay system containing NADP⁺, ATP, glucose-6-P dehydrogenase, and a P-glucose isomerase-free preparation of hexokinase. Glucosone was prepared from glucosazone as described by Becker and Day (8). It was free from glucose or fructose. 6-Deoxyglucose was prepared by mild acid hydrolysis of its β-methyl glycoside (Pierce Chemical Company). Glucosone, glucosamine, mannose, and 2-deoxyglucose were estimated by measuring ADP formation following reaction with ATP and yeast hexokinase.

Mannose-6-P was prepared by incubating hexokinase, ATP, and glucose-free mannose; it was isolated from the reaction mixture as an alcohol-insoluble barium salt and subsequent column chromatography on anion exchanger AG-21K (Bio-Rad) with 0.1 N HCl for elution. It was free from ADP; mannose-6-P was

standardized as mannose after treatment with alkaline phosphatase from *Escherichia coli*.

ADP used for product inhibition experiments was freed from contaminating ATP by incubating with the required amount of glucose, excess of NADP⁺, glucose-6-P dehydrogenase, and hexokinase; after ATP was consumed the mixture was heated at 60° at pH 3.5 for 35 min and centrifuged, following which no hexokinase activity reappeared on neutralization of the supernatant to pH 7.4. All of the solutions of ADP, ATP, and glucose were standardized before use.

All other substrates, nucleotides, and enzymes were from Boehringer.

Protein Estimation—Protein was estimated by the method of Lowry *et al.* (9) with internal standards of bovine plasma albumin. In some cases the method of Warburg and Christian was used (10).

RESULTS

Purification of Glucokinase from Hexokinase-negative Mutant

Washed, freshly harvested cells of 7L1, 65 g, obtained from 3.5 liters of yeast extract-peptone-glucose medium were suspended in 55 ml of a buffer containing 50 mM potassium phosphate (pH 7.4), 2 mM EDTA, and 2 mM β-mercaptoethanol. This was crushed in a French pressure cell and centrifuged at 12,000 × *g* for 5 min, the debris was washed with a further 30 ml of this buffer, and the combined supernatant was centrifuged at 20,000 × *g* for 10 min. The supernatant (90 ml) was treated with DNase (0.04 mg per ml) and RNase (0.05 mg per ml) for 30 min in an ice bath and then treated with 25 g of solid (NH₄)₂SO₄ to bring to 0.45 saturation; pH was maintained at 7.4 during the process. The precipitate was discarded and the supernatant (100 ml) was brought to 0.55 saturation by adding 6.5 g of (NH₄)₂SO₄. The resulting precipitate containing nearly all of the glucokinase activity was dissolved in 5 ml of a buffer containing 10 mM potassium phosphate (pH 7.4), 1 mM β-mercaptoethanol, 1 mM EDTA, and 2 mM glucose and was dialyzed overnight against this buffer with several changes.

The dialyzed solution containing some inactive precipitate was centrifuged and then adsorbed on a DEAE-cellulose column (32-cm length and 80-ml packed volume) previously equilibrated with the above dialysis buffer. The column was washed with 400 ml of this buffer and subsequently eluted with a 500-ml linear gradient of 0 to 0.6 M KCl dissolved in the same buffer. Samples of 2.5 ml were collected per 10-min interval. Maximal enzyme activity was eluted at 0.24 M KCl. Fractions containing more than 2 units per ml and corresponding to the span of 0.22 to 0.28 M KCl were combined and treated with solid (NH₄)₂SO₄ to bring to 0.55 saturation. The precipitate was centrifuged, dissolved in 50 mM Tris-1 mM β-mercaptoethanol-1 mM EDTA-2 mM glucose (pH 7.45), and dialyzed as before against this buffer.

The dialysate was centrifuged to remove inactive proteins and adsorbed on a DEAE-Sephadex A-25 column (2 g of dry resin; 15-cm length) equilibrated with the Tris dialysis buffer. The column was washed with 65 ml of this buffer until a linear gradient of 100 ml of 0 to 0.3 M KCl in the same buffer was applied; fractions of 2.8 ml were collected per 10-min interval. The fraction containing maximal activity appeared when the gradient became 0.13 M KCl; fractions eluted with 0.11 to 0.15 M KCl were pooled and reprecipitated with 0.55 saturation of (NH₄)₂SO₄. The precipitate was dissolved in 10 mM potassium phosphate-1 mM EDTA-1 mM β-mercaptoethanol buffer (pH 7.4),

TABLE I

Summary of purification procedure for glucokinase from hexokinaseless mutant 7L1

Details are as in text.

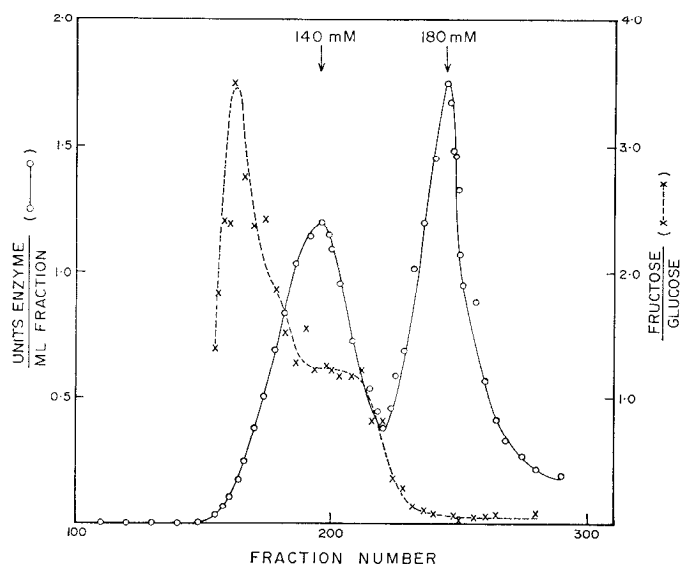| Fraction	Volume	Total gluco-kinase	Protein	Specific activity	Yield
	ml	*units*	*mg/ml*	*units/ mg*	*%*
Crude extract	90	530	29.5	0.2	
0.45–0.55 saturation (NH₄)₂SO₄ precipitate	5	510	60.0	1.7	96
DEAE-cellulose fraction in (NH₄)₂SO₄	3	270[a]	11.2	8.0	51
DEAE-Sephadex fraction in (NH₄)₂SO₄	1.5	147[a]	4.6	21.3	28

[a] Most of the losses occur during dialysis.

FIG. 1. Separation of glucokinase and hexokinase of *S. cerevisiae* by column chromatography of an $(NH_4)_2SO_4$ fraction on DEAE-cellulose. Glucose-grown wild type haploid yeast, 24 g, wet weight, was crushed in 35 ml of 50 mM potassium phosphate buffer, pH 7.4, containing 2 mM β-mercaptoethanol and 2 mM EDTA. The 20,000 × *g* supernatant was processed as described under "Results." The fraction precipitating between 0.45 and 0.55 saturation of $(NH_4)_2SO_4$ was taken up in 3 ml of 10 mM potassium phosphate buffer containing 1 mM EDTA and 1 mM β-mercaptoethanol. The ratio of activity with glucose relative to that of fructose was 0.6 in the crude extract and 1.7 in the enriched fraction. This was dialyzed overnight against the above buffer and fractionated in a DEAE-cellulose column (48 cm long; 44-ml packed volume) with a linear gradient of 500 ml of 0 to 0.3 M KCl dissolved in the same buffer as above but containing in addition 1.5 mM glucose-free fructose. The flow rate was 7 ml per hour, 1.1- to 1.2-ml fractions being collected. The *numbers* at the *top* of the figure indicate the concentration of KCl at the peak positions. The quotient *fructose/glucose* indicates the relative velocity of fructose phosphorylation to that of glucose. Samples corresponding to 2 to 25 μl were used per assay. *Enzyme* refers to glucose-phosphorylating activity.

dialyzed against this buffer, and stored over crushed ice. Table I shows a summary of the purification procedure.

To rule out the possibility that glucokinase activity was derived from hexokinase by proteolytic action of some enzyme present in the hexokinaseless mutant, crude cell extracts of wild type *S. cerevisiae* were mixed with an extract from the mutant 7L1. Periodic examination of this mixture for relative rates of fructose and glucose phosphorylation revealed no change in the relative rate over a period of several hours either at 23° or at 0°. These results also show that the lack of fructose phosphorylation was not due to the presence of any inhibitor in the mutant extract.

Glucokinase from Wild Type S. cerevisiae

$(NH_4)_2SO_4$ fractionation of crude cell extracts from the wild type yeast was used to enrich for glucokinase which was found to precipitate nearly completely between 0.45 and 0.55 saturation of $(NH_4)_2SO_4$ while hexokinase required 0.7 saturation under these conditions. Results in Fig. 1 describe the DEAE-cellulose chromatography of such an enriched fraction. The enzyme activity in the first active fraction phosphorylated fructose at a rate 1.2 times as fast as it did with glucose, while that in the second phosphorylated glucose 20 times as fast as with fructose.

TABLE II

Phosphorylation of various sugars by yeast hexokinase, wild type Fractions I and II, and glucokinase from mutant

The Michaelis constant (K_m) and relative maximum velocity (V_{max}) were determined from initial velocity measurements with the reciprocal plot (12) with a minimum set of six different sugar concentrations. K_m is expressed as millimolar concentration and V_{max} was calculated with respect to glucose. For glucose and fructose, the coupled assay with glucose-6-P dehydrogenase was used and for the rest ADP assay as described under "Experimental Procedure." The ATP concentration was 2.5 mM in all of these experiments. The specific activities toward glucose of Fraction I, Fraction II, and the glucokinase from the mutant were 5.2, 7.3, and 20.5 units per mg of protein, respectively.

Substrate	Yeast hexokinase[a]		Fraction I		Fraction II		Glucokinase from mutant	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
Glucose	0.1	100	0.08	100	0.03	100	0.03	100
2-Deoxyglucose	0.3	100	0.85	103	1.21	46	1.46	45
Mannose	0.05	80	0.11	47	0.24	27	0.12	20
Glucosamine	1.5	70	1.0	50	0.6	15	0.74	9
Glucosone	0.02	20					0.01	10
Fructose	0.7	180					31	0.4

[a] Taken from Sols *et al.* (11).

The earlier eluted portion of the first active fraction seemed to have a much higher fructose to glucose ratio. Whether this was due to any minor enzyme catalyzing fructose phosphorylation has not been determined. The contents of 11 tubes (numbered 190 to 200) representing hexokinase and those of seven tubes (numbered 242 to 248) representing glucokinase were pooled separately, precipitated with $(NH_4)_2SO_4$ to 0.8 saturation, and centrifuged, and the precipitate was collected in 10 mM potassium phosphate (pH 7.4), 1 mM β-mercaptoethanol, and 1 mM EDTA. The specific activity of these fractions toward glucose was 5.2 (Fraction I) and 7.3 (Fraction II) units per mg of protein, respectively.

Properties of Glucokinase

Contaminating Enzymes—The purified glucokinase from the mutant was found to be free from detectable levels of most contaminating enzymes which might interfere with the studies reported here. The following is a list of the various enzymes assayed, the figures within parentheses giving the upper limit of activity expressed as percentage of the glucokinase activity: adenylate kinase (0.5), pyruvate kinase (1.1), ATPase (1.0), 6-P-gluconate dehydrogenase (0.1), P-glucomutase (0.1), P-glucose isomerase (0.4), P-mannose isomerase (0.2).

Substrate Specificity—A comparison of substrate specificity of the glucokinase from the mutant and from the wild type (Fraction II) is indicated in Table II. It can be seen that the kinetic constants for these two preparations are quite similar within errors of such measurements. Table II also shows the substrate specificity of the enzyme in Fraction I. Comparison of these data with those obtained by using purified yeast hexokinase (11) shows the over-all similarity between these later two enzymes except in their affinity to 2-deoxyglucose.

Other sugars and sugar derivatives which at 10 mM concentration failed to produce ADP from ATP when incubated with the enzyme from the mutant are: fucose, 6-deoxyglucose, galactose,
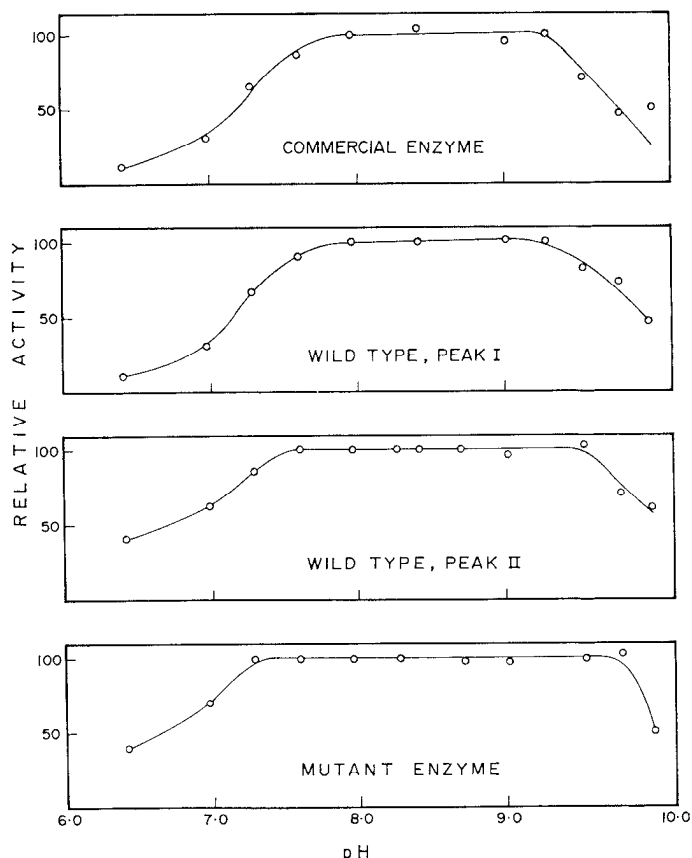
FIG. 2. pH profile of the activity of commercial yeast hexo-kinase, wild type yeast hexokinase (Fraction I, labeled here as *Peak I*), wild type yeast glucokinase (Fraction II, labeled here as *Peak II*), and glucokinase from the mutant. The assay was done by measuring glucose-6-P formation as described under "Experimental Procedure" except that buffers of varying pH values were used and ATP concentration was 2.5 mM. Adjustment of pH was made in presence of added 10 mM $MgCl_2$ in 50 mM buffer with a Radiometer pH meter 4. The pH stayed constant to 0.005 pH unit immediately after the assay. Barbital buffer was used between pH 6.41 and 9.01; glycine buffer was used at higher pH values. An amount of enzyme giving 2 to 10 milliunits of activity at the particular pH was used for each assay. The rates were zero order with respect to $NADP^+$ and glucose-6-P dehydrogenase. Observed velocities were normalized with respect to that at the optimum pH.

arabinose, ribose, gluconic acid, N-acetylglucosamine, and sucrose. The amount of enzyme used was such that a measurable rate would have been obtained if any of these would react at a rate 0.5% of that of glucose.

The phosphoryl donor most effective for glucokinase with glucose as an acceptor was ATP. Nucleoside triphosphates at 2 mM levels gave relative rates of phosphorylation as: ATP, 100; GTP, 3.7; CTP, 2.0; and UTP, 1.2. The following compounds were inactive as phosphoryl donor to glucose: pyrophosphate, P-enol pyruvate, creatine-P, acetyl-P, ADP, glucose-1-P, fructose-6-P, and fructose-1,6 di-P. The K_m for ATP at 10 mM glucose was 48 and 53 μM, respectively, at pH 7.4 (triethanolamine) and pH 8.0 (Tris) under the conditions given earlier.

pH Optimum—The glucose-phosphorylating activities of glucokinase from the mutant and wild type and the wild type hexokinase (Fraction I) were compared with that of the com-
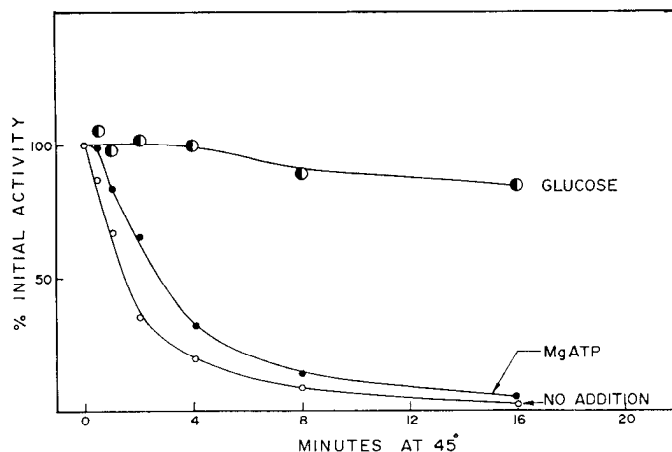

FIG. 3. Effect of glucose on the heat inactivation of glucokinase. Mutant glucokinase (specific activity, 20 units per mg of protein), 0.1 mg, was diluted in 50 mM potassium phosphate buffer, pH 7.4, containing either 2 mM glucose or 5 mM Mg-ATP or nothing, was heated at 45° for the intervals indicated, after which samples were quickly chilled in ice. Results are expressed as percentage of the unheated control.

mercially available yeast hexokinase as a function of pH. Barbital and glycine buffers were chosen, as in the pH range between 8.7 and 9.0 both gave identical rates. Results are given in Fig. 2. The pH optimum for glucokinase from the mutant and that from the wild type was even broader than that of hexokinase and lay between pH 7.5 and pH 9.6. The other feature of the pH velocity relationship was that at pH 6.4 both the hexokinase from commercial source and Fraction I from the wild type haploid yeast were only 10% as active as at their pH optima, whereas the glucokinase from the mutant and from the wild type (Fraction II) was 40% as active. We have not tested, however, whether the decreased velocity on either side of the pH optima was due to decreased affinity for the substrates.

Stability—Purified yeast glucokinase, either from the mutant or from the wild type, was a fairly stable enzyme at 0° in the presence of $(NH_4)_2SO_4$. In dilute solution containing 0.2 mg of protein per ml or less, it lost half of the activity in 24 hours either at 0° or 24°. In the frozen state 90% of the activity was lost in 12 hours. Glucose protected the enzyme from thermal inactivation (Fig. 3); protection by Mg-ATP, if any, was marginal. Sodium or potassium ions had no effect on either activity or stability of the enzyme. This is unlike rat liver glucokinase which needs potassium ions for stability (4).

Electrophoretic Mobility—The purified enzyme from the mutant on being subjected to disc electrophoresis in polyacrylamide gel in Tris-glycine buffer, pH 8.8, by a modification of the method of Davis (13) and subsequent development with the assay solution containing glucose, ATP, glucose-6-P dehydrogenase, $NADP^+$, phenazine methosulfate, and nitro blue tetrazolium (14) showed a broad single band that reacted with ATP and glucose to produce glucose-6-P. The band also reacted with fructose, but very slowly. A band of the same characteristics was observed when crude extracts of the mutant 7L1 or from other hexokinaseless mutants were used. Fraction II from wild type showed the same band that was glucose-positive and fructose-negative. It had not been possible to obtain a sharp hexokinase band under these conditions with either Fraction I
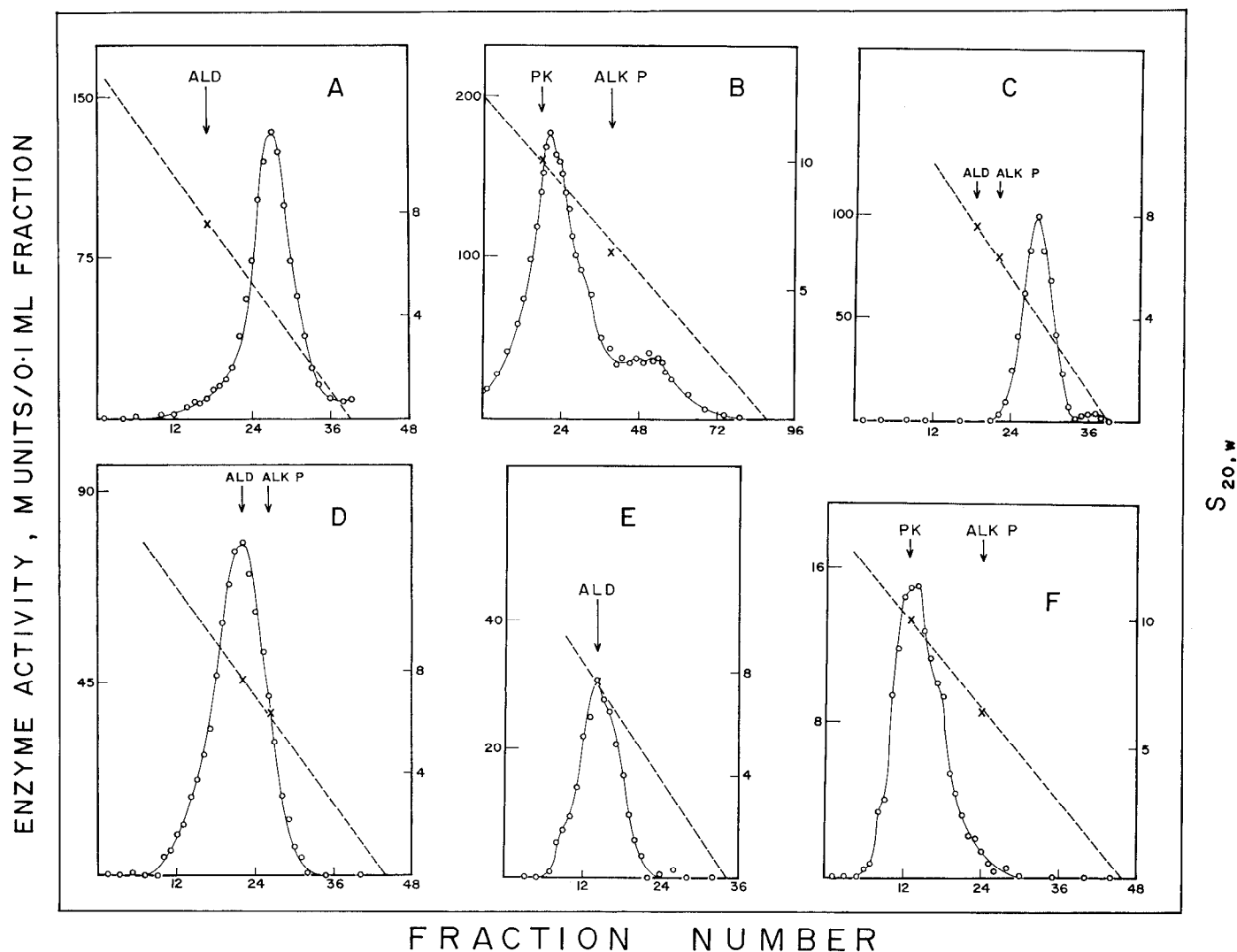
FIG. 4. Sedimentation velocity profile of yeast hexokinase and glucokinase in a 5-ml sucrose density gradient. All gradients except that in Experiment *F*, were in 10 mM phosphate buffer; in Experiment *F*, 50 mM Tris buffer was used. In Experiment *B*, 2 mM glucose-free fructose replaced glucose. Activity was expressed in milliunits per 0.1 ml of fraction. *A*, unfractionated crude extracts of glucose-grown wild type haploid *S. cerevisiae* equivalent to 0.1 mg of protein. *B*, a glucokinase-enriched fraction of the same extracts as in *A* and fractionated in presence of 2 mM phenylmethanesulfonyl fluoride (19) to prevent proteolysis; an $(NH_4)_2SO_4$ fraction precipitating between 0.45 and 0.53 saturation having a glucose to fructose ratio of 4.2 and containing 5 units of glucose-phosphorylating activity was used. *C*, fractionated

hexokinase Fraction I from wild type equivalent to 0.8 unit of activity. *D*, fractionated glucokinase Fraction II from wild type containing 0.8 unit of activity. *E*, unfractionated crude extracts of glucose-grown culture of the mutant 7L1 containing 0.7 mg of protein. *F*, 0.2 unit of purified mutant glucokinase prepared in the presence of 2 mM phenylmethanesulfonyl fluoride (19). The *dotted line* joining the $s_{20.w}$ values of the marker enzyme cuts the *x*-axis at the meniscus ($s_{20.w} = 0$). Fractions are numbered beginning from bottom of the tubes. The *left-hand side markings* on the *ordinates* refer to the enzyme activity and the *right-hand side markings* to the sedimentation constant ($s_{20.w}$). *ALD, ALK P,* and *PK* indicate aldolase, alkaline phosphatase, and pyruvate kinase, respectively.

or crystalline commercial yeast hexokinase. Instead, a very diffuse zone of enzyme was observed. When crude extract of the wild type haploid *S. cerevisiae* was used, the band due to glucokinase was easily visible, but hexokinase was indicated only by a broad fast moving zone. The crude extract of the mutant 7L1 did not yield this diffuse zone of enzyme activity. No attempt was made to locate other protein bands on the purified glucokinase.

Sedimentation Profile—In order to get an idea of the approximate molecular weight of yeast glucokinase, sucrose density gradient studies were performed by the method of Martin and

Ames (15). The gradient was linear between 5 and 20% sucrose in either 10 mM potassium phosphate or 50 mM Tris, each containing 1 mM β-mercaptoethanol, 1 mM EDTA, and 2 mM glucose or fructose at pH 7.4. Each tube contained one or more of the marker proteins, *E. coli* alkaline phosphatase, rabbit muscle aldolase, and rabbit muscle pyruvate kinase; their $s_{20.w}$ values are 6.3 (16), 7.6 (17), and 10.0 (18), corresponding to molecular weights 86,000, 144,000, and 237,000, respectively. The enzyme solution together with the marker enzyme in 0.2 ml of the buffer in which the gradient was made was layered on top of the gradient. This was centrifuged at 36,000 to 37,000 rpm for 15 to 17
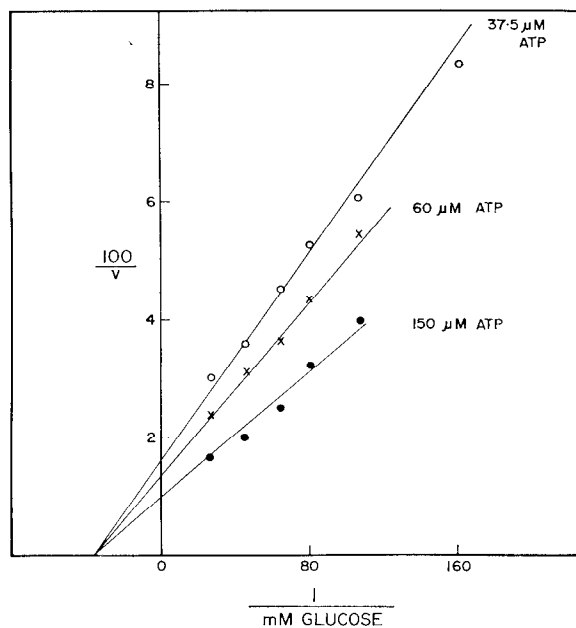
FIG. 5. Double reciprocal plot showing the effect of glucose concentration on glucokinase velocity (V) at a number of ATP concentrations. Experiments were done in 10 mM MgCl$_2$ and non-limiting amounts of NADP$^+$ and glucose-6-P dehydrogenase as described earlier; glucose and ATP concentrations are as indicated. Velocity is expressed in arbitrary fluorescence units. The amount of enzyme used per assay was an equivalent of 36 milliunits. The x-axis intercept gives a K_{glucose} of 28 μM.
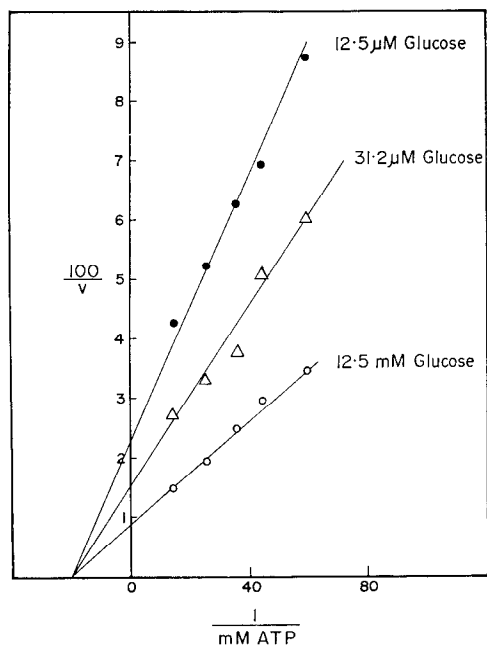

FIG. 6. Double reciprocal plot showing the effect of ATP on glucokinase velocity (V) at various glucose concentrations. The amount of enzyme per assay was equivalent of 48 milliunits. Other conditions were as in Fig. 5. The x-axis intercept gives a K_{ATP} of 50 μM.

hours at 0–2° in a Beckman model L ultracentrifuge with an SW-39 rotor. Nearly 40 2-drop fractions were collected in all cases except one (Fig. 4B) and these were assayed for glucose-phosphorylating activity by measuring glucose-6-P formation
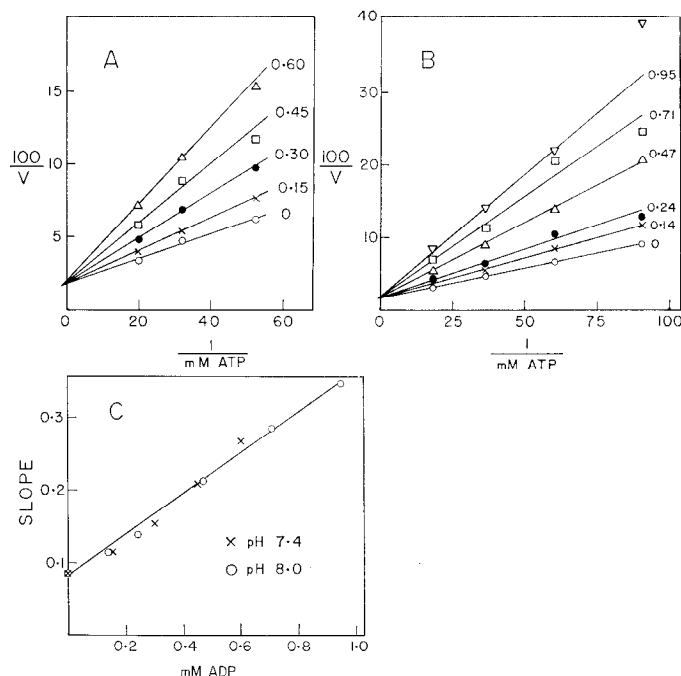

FIG. 7. Plot of reciprocal of initial reaction velocity (V) of yeast glucokinase against reciprocal of the millimolar concentration of ATP at various ADP concentrations indicated on the graph at pH 7.4 (A) and pH 8.0 (B). Numbers against each *line* refer to the millimolar concentration of ADP. Tris buffer, 50 mM, was used at pH 8.0. The amounts of enzyme per assay were 25 milliunits at pH 7.4 and 12 milliunits at pH 8.0. Glucose concentration was 12 mM in A and 10 mM in B. All other details are the same as in Fig. 5. C is a plot of slopes of lines in A and B against the respective ADP concentrations.

as described earlier. In the experiment shown in Fig. 4B, 87 1-drop fractions were collected. Recovery of glucokinase was of the order of 90%.

Results of these experiments (Fig. 4) indicate that yeast glucokinase sediments faster than yeast hexokinase. Either in crude extracts or in purified Fraction I (Fig. 4, A and C) yeast hexokinase gave an $s_{20,w}$ value of 4.3 and 4.1, respectively, indicating dissociation to half-molecules in presence of phosphate and glucose (20, 21). On the other hand, glucokinase from crude extracts of the mutant and from the wild type (Fraction II) sedimented together with aldolase (Fig. 4, E and D); unlike aldolase, which gave a symmetrical peak (not shown), the sedimentation profile of glucokinase suggests heterogeneity with respect to a heavier species. This heterogeneity is evident in Fig. 4, B and F, where an $s_{20,w}$ value of nearly 10 is indicated. Under these conditions both of the reference proteins, pyruvate kinase and alkaline phosphatase, gave symmetrical peaks. Results in Fig. 4B further indicate that, when the crude extract of the wild type yeast was fractionated with (NH$_4$)$_2$SO$_4$, the fraction phosphorylating glucose faster than fructose yielded a component that sedimented more rapidly than hexokinase. In unfractionated extracts, however, the faster sedimenting component was masked by the preponderance of hexokinase activity (Fig. 4A).

Kinetic Studies

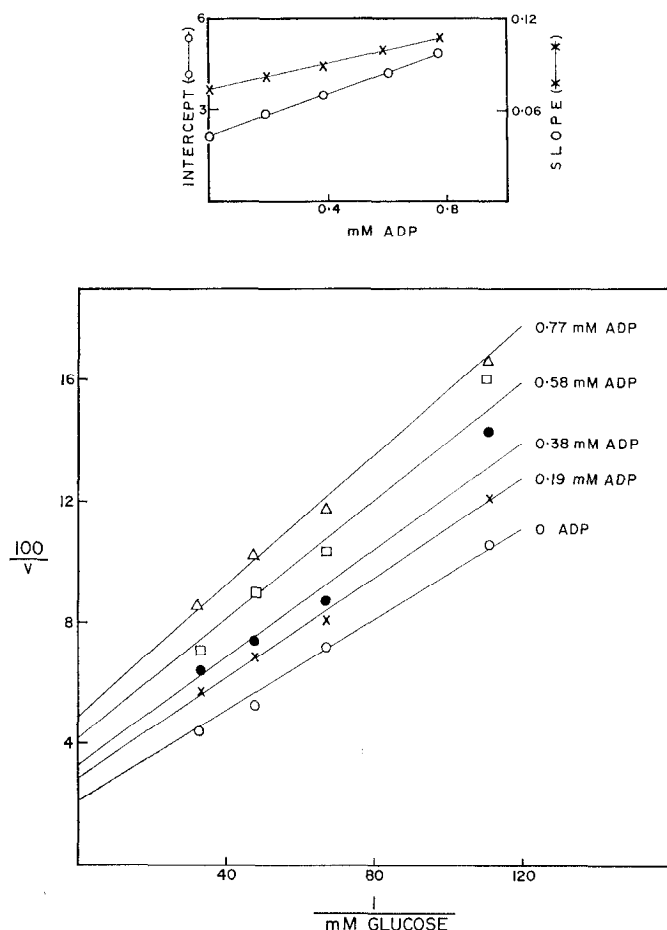For all experiments described in this section, glucokinase from the mutant was used.

FIG. 8. Double reciprocal plot of reaction velocity (V) of yeast glucokinase and glucose concentration at varying levels of ADP. The concentration of ATP in all of the assays was held constant at 1.3 mM. ADP used was free of glucose. The amount of enzyme per assay corresponded to approximately 24 milliunits. All other details are the same as in Fig. 5. The *upper figure* is a secondary plot of slopes and ordinate intercepts against the respective ADP concentration.

Glucose-ATP Relationship—To determine whether the affinity of glucokinase for glucose was a function of the concentration of ATP or that of ATP a function of glucose, experiments described in Figs. 5 and 6 were carried out. It is seen from these results that the double reciprocal plots for velocity against substrate concentrations are linear over the ranges studied and all of the lines meet at a common point on both 1/glucose and 1/ATP axes. The observed independence of the K_m values for glucose and ATP of the concentrations of ATP and glucose, respectively, suggested that the substrates did not compete for binding to the enzyme. Secondary plots of y-axis intercepts from Figs. 5 and 6 against the reciprocals of the respective fixed substrate concentrations yielded straight lines; the K_{ATP} and $K_{glucose}$ values calculated from x-axis intercepts of such secondary plots gave values of 41 and 27 μM, respectively, in reasonable agreement with those from Figs. 6 and 5.

Inhibition by Products, Sugar, and Sugar Analogue—In addition to the sugar analogue N-acetylglucosamine, small concentrations of ADP inhibit yeast glucokinase activity considerably. The inhibition by ADP was investigated in some detail in order to elucidate some aspects of the reaction mechanism. The maximal adenine nucleotide concentration in these experiments never exceeded 4 mM. To ensure that variation in ATP and

TABLE III

Inhibition characteristics of yeast glucokinase

Double reciprocal plots were used to evaluate inhibition data. Unless otherwise mentioned, ATP concentration was 1 mM.

Inhibitor	Inhibitor concentration	Variable substrate	Nature of inhibition	K_i	Reference
	mM			mM	
ADP	0–0.95	ATP	Competitive	0.3	Fig. 7
	0–0.77	Glucose	Noncompetitive	0.6[a]	Fig. 8
				1.6[b]	
N-Acetylglucosamine	0, 1.25, 2.5	Glucose	Competitive	1.7	c
	0, 0.5, 1.0	Fructose	Competitive	1.2	c
Fructose	0, 10, 20	Glucose	Competitive	53.5	c

[a] K_i intercept.
[b] K_i slope.
[c] Data not shown.

ADP concentrations resulted in changes in their magnesium complexes, 10 mM $MgCl_2$ was used in all studies. At 1 mM ATP, Mg^{++} concentrations up to 12 mM did not inhibit the activity; 20 mM Mg^{++} under these conditions decreased velocity no more than 3%. Similar results were obtained at an ATP concentration equal to its K_m. This made unlikely the possibility that free ATP was the substrate.

Figs. 7 and 8 describe the results of ADP inhibition experiments. It can be seen from these graphs (Fig. 7, A and B) that ADP acted in competition with ATP on the enzyme, affecting primarily the slopes and not the y-intercept of these lines at either pH 7.4 or at pH 8.0. The concentration of the other substrate, glucose, was held constant at a value nearly 300 times its K_m and the enzyme was probably saturated with this substrate. The secondary plot of slopes of these lines against the inhibitor concentration (Fig. 7C) described a straight line, showing that ADP inhibition was linear competitive with a K_i of 0.3 mM at either pH.

When similar experiments were performed with glucose as the variable substrate, the results indicated a noncompetitive relationship between ADP and glucose (Fig. 8). Both the slopes and y-intercepts varied. The lines met at a point below the horizontal axis, suggesting that K_i slope was greater than K_i intercept. The inhibition was linear as indicated by a replot of the slopes and y-intercepts against ADP concentrations. The x-axis intercepts of the secondary plot gave a K_i slope of 1.65 and K_i intercept 0.58 mM ADP. Similar experiments at pH 8.0 confirmed the linearity of the inhibition up to an ADP concentration of 2.5 mM.

Regarding the other product, sugar phosphate, the following is a summary of the results of preliminary experiments. When both glucose and ATP were limiting (equal to their respective K_m values) 2 mM glucose-6-P caused less than 50% inhibition. Mannose-6-P behaved as a partially competitive inhibitor to glucose. The inhibition appeared to be saturating at 0.5 mM mannose-6-P at 1.5 mM ATP. When ATP was the variable substrate and glucose was saturating (10 mM), mannose-6-P failed to inhibit yeast glucokinase. AMP was a weak inhibitor; with ATP as the variable substrate, AMP gave nonlinear reciprocal plots at saturating concentrations of glucose. Table III summarizes the results of experiments with a number of inhibitors.

Control of Glucokinase Formation

The steady state differential rates of glucokinase appearance in the mutant 7L1 in the yeast extract-peptone medium supplemented with acetate, fructose, and glucose were, respectively, 3.0, 3.0, and 4.9 milliunits per unit increase in yeast mass measured as extinction at 650 mμ. Galactose was found to be similar to glucose in this regard. When such experiments were performed with the wild type yeast, hexokinase was found to be produced at a 50% higher rate in the presence of glucose over that in a basal medium devoid of added glucose. The semi-constitutive nature of the synthesis of both glucokinase and hexokinase is indicated.

DISCUSSION

The sugar substrate specificity of yeast glucokinase is broader than that of *Aerobacter* or hepatic enzymes (1, 3, 4) in that it phosphorylates mannose, 2-deoxyglucose, and glucosamine. The yeast enzyme, is however, characterized by a relatively high rate and affinity for glucose which makes the phosphorylation coefficient[1] (11) for all sugars except glucosone much less than that for glucose. The requirement on the glucose skeleton of an aldehyde group at C-1 and a hydroxymethyl at C-5 appears to be essential; at C-2 the binding requirement is less specific.

A comparison of the specific activity of crude cell-free extracts from the wild type and from the mutant 7L1 indicates that glucose-phosphorylating activity in the mutant is 10 to 15% of that of wild type. This estimate of the relative amount of glucokinase and hexokinase in the wild type *S. cerevisiae* is only approximate. The 2-deoxyglucose resistance of the mutant appears intriguing; while the wild type parent fails to grow in the salt-vitamin-galactose medium containing 0.3 mM 2-deoxyglucose, the mutant 7L1 grows normally in the presence of 50 mM concentration of this substance despite its reactivity to glucokinase (Table II). Earlier studies by Heredia, De La Fuente, and Sols (22) indicated that the inhibitory effects of 2-deoxyglucose on growth and fermentation of baker's yeast arise from its phosphorylation to 2-deoxyglucose-6-P. The hexokinaseless mutant used in these studies, unlike its wild type parent, was unable to take up any detectable amount of 2-deoxyglucose from an aerated suspension containing an oxidizable energy source and 0.3 mM 2-deoxyglucose. It appears, therefore, that the glucokinase activity in the mutant is insufficient to bring about phosphorylation of this sugar analogue to a significant extent.

Sedimentation velocity profile of yeast glucokinase indicates that its minimum molecular weight is approximately the same as that of aldolase (144,000). However, the enzyme does not sediment as a single symmetrical peak in the sucrose density gradient, as does hexokinase (Fig. 4, *A* and *C*). In dilute solution such as in crude extracts (Fig. 7E) the bulk of the enzyme cosediments with aldolase, although a heavier component of an $s_{20,w}$ of nearly 10 is apparent, indicating a molecular weight well in excess of 200,000. When, however, the enzyme is treated with phenylmethanesulfonyl fluoride during its preparation, as shown in Fig. 4, *B* and *F*, the major sedimenting component has an $s_{20,w}$ value of 9.6. This is interesting, because there is no detectable kinetic or electrophoretic difference between gluco-

[1] Phosphorylation coefficient

$$= \frac{V_{max} \text{ for substrate}}{V_{max} \text{ for glucose}} \times \frac{K_m \text{ for glucose}}{K_m \text{ for substrate}}$$

kinase preparations obtained in absence and in presence of this proteolytic inhibitor. Components even heavier than 9.6 $s_{20,w}$ appear to be present in such preparations (Fig. 4F). When such a purified preparation as in Fig. 4F was sedimented in 10 mM phosphate, a marked tendency toward aggregation was noticed. The observation of Rustum and Ramel (5) on a species of baker's yeast glucokinase of molecular weight 1.2 × 10⁶ possibly refers to an aggregated form of this enzyme. The effect of glucose on the sedimentation behavior could not be studied as the sucrose gradient was found to be contaminated with traces of glucose.

The kinetic data described in Figs. 5 to 8 indicate a close similarity of yeast glucokinase with yeast hexokinase as reported by Fromm and Zewe (23). The results in Figs. 5 and 6 on the constancy of the K_m values for glucose and ATP irrespective of the variation in the concentration of ATP and glucose, respectively, suggest that the reaction between glucose, ATP, and glucokinase takes place in a random sequence in which none of the steps prior to the interconversion of enzyme-ATP-glucose and enzyme-ADP-glucose-6-P is rate-controlling. This observation, together with the observed inhibition data with ADP, helps to suggest the reaction mechanism as one of rapid equilibrium random with a dead end ternary complex between the enzyme, glucose, and ADP (24). Binding studies with pure preparations of the enzyme and kinetic characteristics of the back reaction should serve to confirm the proposed mechanism.

Our initial attempts have failed to reveal any striking control property of yeast glucokinase. Sugar phosphates in physiological concentrations do not inhibit the enzyme strongly. Although the other product, ADP, is a fairly potent inhibitor, the reversal of the inhibition by ATP suggests that the ATP:ADP ratio could serve at best as a marginal control parameter. The simultaneous presence of ADP and mannose-6-P in the presence of low concentrations of glucose and ATP inhibits glucokinase no more than either of them would singly do. Similar properties of yeast hexokinase have been observed (23). Thus, the question of control of glucose utilization in yeast remains largely unanswered. It remains to be seen what role Mg^{++}-chelating agents such as citrate would have in the control of this important process.

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