Heart Muscle Hexokinase: Subcellular Distribution and Inhibition by Glucose 6-Phosphate[1]

STEVEN E. MAYER,[2] ANNETTE C. MAYFIELD, AND JOSEPH A. HAAS[3]

Department of Pharmacology, Division of Basic Health Sciences, Emory University, Atlanta, Georgia 30322

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SUMMARY

The intracellular distribution of hexokinase and the susceptibility of partly purified preparations of the enzyme to inhibition by glucose 6-phosphate was determined in the hearts of several species. The use of sucrose as a medium for fractionation of heart homogenates indicated that most of the enzyme was nonparticulate, but on differential centrifugation in isotonic KCl a large fraction of hexokinase activity was associated with mitochondria.

To test the hypothesis that glucose utilization is controlled through the inhibition of hexokinase by glucose-6-P, enzyme activity from heart mitochondria and soluble fraction was measured under conditions in which glucose-6-P was or was not allowed to accumulate. The apparent inhibitor constants for glucose-6-P ranged from 3×10^{-5} to 1.3×10^{-4} M in various preparations, and this inhibition was noncompetitive relative to ATP or glucose. However, aging of the enzyme prepared from dog heart resulted in a 3- to 10-fold increase in the apparent K_i, and an increase in the K_m of ATP and the interaction between glucose-6-P and ATP became competitive. A change in the activity-pH relationship of hexokinase also occurred. Inorganic orthophosphate antagonized the inhibitory effect of glucose-6-P in concentrations comparable to physiological ones. This effect was lost on aging of dog heart mitochondria.

The data on the *in vitro* kinetics of heart hexokinase agree with observations of others on the control of glucose phosphorylation in perfused heart under conditions in which relatively large changes in glucose-6-P and inorganic phosphate concentrations occur such as after the administration of epinephrine and during anoxia, respectively. They are also in agreement with the fact that the administration of large doses of epinephrine to intact animals results in an increase in cardiac glucose 6-phosphate concentration and accumulation of intracellular glucose, presumably as a result of inhibition of hexokinase activity.

INTRODUCTION

Regulation of glucose utilization in muscle tissues has been demonstrated at three steps: membrane transport (1), glucose phosphorylation and the phosphorylation of fructose-6-P (2). Control at the second step is thought to be the result of hexokinase inhibition by glucose-6-P (2, 3). That glucose phosphorylation is inhibited when glucose-6-P accumulates has been demonstrated in intact cells (2, 4, 5). Furthermore, the accumulation of intracellular glucose during epinephrine-induced increase in plasma glucose and free fatty acids has been related to glucose-6-P ac-

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cumulation secondary to the stimulation of glycogenolysis (6) and possibly also to the inhibition of phosphofructokinase by fatty acids (7). Thus, an increase in the concentration of cardiac glucose-6-P and the subsequent inhibition of glucose phosphorylation could be an important consequence of the effect of hormones, such as epinephrine and glucagon, on cardiac glycogenolysis and of epinephrine on lipolysis.

While it has been established that glucose-6-P is a potent inhibitor of heart hexokinase (8), a detailed analysis of the kinetics of this enzyme has not been reported. The purpose of this paper is to relate such information to the apparent role of hexokinase as a control point in glucose utilization. Further, these experiments were designed to permit the study of the kinetics of the enzyme under conditions of minimal loss of activity. This was done in order to test the possibility that alterations in kinetics may occur with purification, since purification has resulted in low recovery of heart hexokinase (9).

Preliminary accounts of this work have been published (10, 11).

METHODS

Enzyme preparations. Preparations for the study of the subcellular distribution of hexokinase and for partial purification of the enzyme were made from the freshly excised hearts of mongrel dogs, guinea pigs, New Zealand albino rabbits, rats (Sprague-Dawley), and turtles (*Pseudemys troostii*). The mammals were anesthetized with sodium pentobarbital, 40 mg/kg, and the turtles were decapitated prior to excision of the hearts. These were immediately placed on ice, minced, and homogenized at 0° in the appropriate medium with a Kontes Duall homogenizer fitted with a motor-driven Teflon pestle. For cell fractionation studies, homogenization and resuspension of sedimented particulate fractions were made with 10 volumes of medium containing 0.25 or 0.88 M sucrose with 10 mM EDTA and 40 mM Tris-HCl pH 7.4 or in a saline medium which contained 0.18 M KCl, 10 mM EDTA, and 40 mM Tris-HCl pH 7.4.

Partial purification of the hexokinase associated with mitochondria was carried out on the pellet which had been sedimented from the saline medium at 15,000 *g* for 30 min at 0°. Subsequent treatment partly followed that of Crane and Sols (9). All operations were carried out between 0 and 4°. The mitochondrial pellet representing 5 g of heart was washed and resuspended twice and then added to 0.1% Triton X-100 (Rohm and Haas) which had been dissolved in the saline medium. This was centrifuged for 30 minutes at 15,000 *g*. The sediment was extracted for 1 hr at 0° in 1.2% Triton and then centrifuged 40 min at 50,000 *g*. The solubilized enzyme preparation was then passed through a 0.5 × 9.5 cm column of Sephadex G-25 (coarse beads), and the enzyme was recovered by elution with the KCl-EDTA-Tris solution containing 5 mM mercaptoethanol. The gel filtration column had been washed with this medium. This step resulted in separation of the enzyme from the detergent. The enzyme preparation containing 100–150 μg protein per milliliter was stored at 2° in the presence of penicillin (10 μg/ml), streptomycin (25 μg/ml), and 5 mM NaF. Some preparations were sterilized by filtration through an 0.45 μ pore Millipore filter and stored without the addition of NaF and antibiotics. Similar results were obtained with both types of preparations. Storage at −20° resulted in rapid loss of enzyme activity.

Partial purification of the soluble fraction of heart hexokinase was carried out on the supernatant solution of saline homogenates which had been centrifuged at 100,000 *g* for 60 min. The supernatant fluid from 5 g of heart was adjusted to pH 5.5 with acetic acid and centrifuged at 34,000 *g* for 10 min. This supernatant solution was adjusted to pH 7.4 with NH_4OH and brought to 66% saturation by the slow addition of saturated ammonium sulfate, pH 7.4, which contained 5 mM mercaptoethanol. After storage overnight at 2° the precipitated enzyme was dissolved in 4 ml 0.18 M KCl, 1 mM EDTA, 5 mM mercaptoethanol, and 50 mM Tris, pH 7.4. In some experiments the enzyme was reprecipitated in 25% saturated am-

monium sulfate, pH 7.4. The final preparation contained 5 mg/ml protein.

Measurement of hexokinase activity. All determinations were made at 30°. The maximum velocity of the reaction was determined under conditions in which no glucose-6-P accumulation could occur. This was done by coupling hexokinase with glucose-6-P dehydrogenase. The medium contained 0.1 M Tris-HCl, pH 7.4, 1 mM glucose, 5 mM nicotinamide, 2 mM neutralized ATP, 0.5 mM TPN^+, 10 mM $MgCl_2$, 1 mg/ml crystalline bovine albumin, 1 mM EDTA, and 1 μg/ml yeast glucose-6-P dehydrogenase in a total volume of 100 μl. The reaction was started by the addition of hexokinase and was measured at 340 mμ in a Beckman monochromator equipped with a Gilford absorbance indicator and cuvette positioner. For the measurement of the K_m of glucose and of ATP the reaction was followed in terms of the fluorescence of TPNH (12) in a Turner Model 111 fluorometer which had been adapted for 0.1-ml cuvettes. With either type of measurement the amount of enzyme added was adjusted so that the reaction rate was linear for at least 5 min at 30°. When determinations were made on crude homogenates or the soluble fraction thereof it was necessary to correct for an approximately 10% excess of TPNH production due to the presence of 6-phosphogluconic acid dehydrogenase.

In experiments in which the inhibition of heart hexokinase by glucose-6-P was to be determined, the reaction was followed by one of two methods. For all but the purified mitochondrial preparation activity was measured in terms of the disappearance of glucose and the accumulation of glucose-6-P. The enzyme preparation was incubated in a total volume of 100 μl for 0–30 minutes in a medium which contained 0.1 M Tris-HCl, pH 7.4, 2 mM ATP, 10 mM $MgCl_2$, and 0.2–1 mM glucose. The reaction was stopped by immersing the tubes in boiling water for 2 min. Glucose-6-P was measured spectrophotometrically according to the method of Hohorst (13) or Lowry *et al.* (14) in a total volume of 60–100 μl. After the reaction had gone to completion (10 min) an aliquot was incubated with 0.3 mM ATP, 6 mM $MgCl_2$, and 2.5 μg/ml crystalline yeast hexokinase for the determination of glucose (14, 15). An experiment was considered to be acceptable only if the amount of glucose which had disappeared was equivalent to the amount of glucose-6-P which had been formed. In all but the purified heart mitochondrial preparations there was a great deal of phosphoglucose isomerase but no phosphofructokinase activity. Therefore, the measured concentration of glucose-6-P was multiplied by 1.25 to account for the equilibrium of the isomerase reaction (16).

Inhibition of hexokinase activity by glucose-6-P could be determined kinetically in purified mitochondrial preparations in which ATPase activity had been reduced to 10% or less than that of hexokinase. The rate of ADP formation was measured by coupling with pyruvate kinase and lactic acid dehydrogenase. The incubation mixture contained 0.1 M Tris-HCl, pH 7.4, 1 mM glucose, 2 mM ATP, 5 mM nicotinamide, 0.3 mM Na_3-phosphopyruvate, 50 mM KCl, 10 mM $MgCl_2$, 3 μg/ml muscle pyruvate kinase and 6 μg/ml Worthington beef heart lactic acid dehydrogenase. DPNH was added to bring the concentration to 0.1 mM after the blank reaction due to the presence of ADP and pyruvate had stopped. The assay was started by the addition of the hexokinase. The reaction rate was linear for at least 10 min at 30°. With purified rat heart mitochondrial hexokinase preparations DPNH oxidizing activity was eliminated by the addition of 1 μg/ml rotenone (17).

Other analytical methods. Unless otherwise stated enzymes were obtained from C. F. Boehringer u. Soehne. Cytochrome oxidase was measured by the method of Brody *et al.* (18), and activity was calculated as the first-order reaction rate (19). Other enzymes determined were: antimycin insensitive cytochrome *c* reductase (20), phosphoglucose isomerase (16) and phosphofructokinase (21). Protein was determined by the method of Lowry *et al.* (22) with the procedure for insoluble protein applied to mitochondria. Reconstituted

human serum was used as the standard (Versatol, Warner-Chilcott).

RESULTS

Subcellular Distribution of Myocardial Hexokinase

Subcellular fractions of hearts were characterized by differential centrifugation (Tables 1 and 2), sucrose density gradient centrifugation (Fig. 1), and light microscopy. While the distribution of cytochrome *c* oxidase and of antimycin-insensitive cytochrome *c* reductase demonstrated the separation of a mitochondrial and a putative microsomal fraction, it is evident from Table 1 that the medium in which centrifugation was performed affected both the fractional distribution and specific activities of these enzymes. Homogenization of heart and resuspension of particulate fractions in 0.28 M sucrose resulted in lower activities of both enzymes in S_1 and in the particulate fractions than when a saline medium was used. Hypertonic (0.88 M) sucrose yielded the same results as did an isotonic solution of the disaccharide. Similar data were obtained on rat and guinea pig hearts.

When the subcellular localization of heart hexokinase was determined by using either differential centrifugation in 0.28 M sucrose (Table 2), 0.88 M sucrose, or a sucrose density gradient (Fig. 1), most of the activity from dog, guinea pig, and rat heart was in the soluble fraction of myocardium. The highest specific activity was found in the 100,000 *g* pellet (Table 2). In rabbit and turtle hearts more of the enzyme was associated with both particulate fractions than was the case with the other species.

When differential centrifugations were made in an isotonic KCl medium the activity of hexokinase in the 600 *g* supernatant fraction was the same as in sucrose, in contrast to the inhibition of the cytochrome enzymes in the latter medium (Table 1). However, Table 2 shows that homogenization in a saline medium resulted in a considerably greater proportion of hexokinase being associated with the mitochondrial fraction along with a decrease in the proportion which was found in the soluble fraction and in the specific activity of the enzyme in microsomes. Relatively little difference in the distribution of rabbit heart hexokinase was noted between homogenization in the saline as compared to the sucrose medium; most of the enzyme

TABLE 1

Characterization of dog heart subcellular fractions by differential centrifugation

Hearts were homogenized in 10 volumes of the appropriate medium, which was also used to resuspend the sedimented fractions. Both media also contained 10 mM EDTA and 40 mM Tris, pH 7.4. Centrifugations were made at 0° in Sorvall SS-34 and Spinco type 40 rotors. Particulate fractions were washed once and centrifuged a second time before assay. Each datum represents the average of at least six experiments.

	Cytochrome oxidase				Cytochrome *c* reductase (antimycin insensitive)			
	0.28 M sucrose		0.18 M KCl		0.28 M sucrose		0.18 M KCl	
Fraction	% S_1	Specific activity[a]	% S_1	Specific activity[a]	% S_1	Specific activity[b]	% S_1	Specific activity[b]
600 *g*, 10 min supernatant (S_1)	100	6.3	100	14.8	100	38.5	100	68.0
15,000 *g*, 30 min supernatant (S_2)	24	1.3	20	3.3	79	28.3	42	21.2
15,000 *g*, 30 min precipitate (P_2)	58	39.9	77	89.4	15	49.6	46	247
100,000 *g*, 1 hr supernatant (S_3)	8	0.84	9	1.5	19	4.0	0	0
100,000 *g*, 1 hr precipitate (P_3)	0	0	5	15.8	44	289	37	388

[a] First-order rate constant per milligram of protein min^{-1} at 30° (19).

[b] Nanomoles of oxidized cytochrome *c* reduced per milligram of protein min^{-1} at 30°.

TABLE 2

Distribution of hexokinase in subcellular fractions of heart prepared by differential centrifugation

Hearts were homogenized and prepared as described in Table 1. Each datum represents the average of six experiments except two for rabbit and turtle.

Fraction	Dog		Rat		Guinea pig		Rabbit		Turtle	
	% S_1	Specific activity[a]	% S_1	Specific activity	% S_1	Specific activity	% S_1	Specific activity	% S_1	Specific activity
	0.28 M sucrose									
S_1	100	20.7	100	29.4	100	70.8	100	49.4	100	102
S_2	84	18.3	96	33.7	87	67.0	63	38.3	67	77.0
P_2	10	18.8	6	13.4	9	46.0	27	60.4	36	120
S_3	65	15.6	88	34.7	73	66.2	19	14.0	12	15.3
P_3	20	119	5	39.8	8	90.1	38	164	27	253
	0.18 M KCl									
S_1	100	26.6	100	29.5	100	69.9	100	46.9	100	98.6
S_2	41	18.0	68	28.7	50	46.9	52	32.3	36	68.1
P_2	40	50.2	30	43.7	46	100	41	72.4	64	141
S_3	28	11.2	52	35.2	33	41.0	16	9.0	0	0
P_3	16	35.3	11	35.2	13	70.9	36	134	34	98.0

[a] Specific activity = nanomoles glucose used per milligram of protein min^{-1} at 30°.

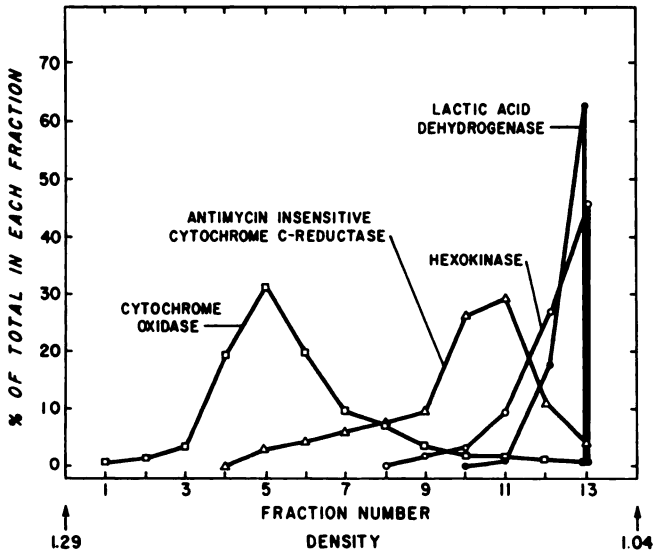

FIG. 1. *Distribution of rat heart enzymes in a sucrose density gradient*

A 0.2 ml sample of S_1 (Table 1) was layered on top of 4.5 ml of a linear sucrose density gradient which ranged in concentration from 0.15 (5%) to 2.25 M (60%) and was prepared according to the method of Martin and Ames (23). The gradient also contained 10 mM EDTA, pH 7.4. The ½ × 2-inch tube was centrifuged for 1 hr at 40,000 *g* in a Spinco SW39L swinging-bucket rotor. Fractions (0.4 ml) were collected from the bottom of the tube.

was associated with particulate fractions in either case. Even with the use of KCl, 52% of rat heart hexokinase remained in the high speed supernatant fraction. On the other hand, all the turtle heart hexokinase was sedimented from the saline medium between 600 and 100,000 *g*.

Thus, the apparent intracellular localization of myocardial hexokinase is a function of the species and the medium which is used for homogenization and fractionation. This is true to a lesser extent of the cytochrome enzymes which were determined (Table 1). In contrast to heart, 78 and 85%, respectively, of rat cerebral cortex hexokinase and cytochrome *c* oxidase sedimented after centrifugation at 12,000 *g* for 15 min in either isotonic sucrose or saline media.

Partial Purification of Heart Hexokinases

A 5- to 6-fold purification of mitochondrial hexokinase was achieved (Table 3).

TABLE 3

Yield of particulate hexokinase from dog heart

Similar results were achieved in purification of the enzyme from hearts of other species except rat. Hearts of the rat contained considerably less particulate enzyme, and the final product was less stable. Details of the purification are given in methods.

Fraction	Specific activity[a]	(%)
Whole homogenate	26.6	100
Mitochondria	50.2	40
0.1% Triton extraction	56.8	37
1.2% Triton extraction	140	30
Eluate from Sephadex G-25	157	30

[a] Nanomoles glucose used per milligram of protein min^{-1} at 30°.

This was adequate for accurate assay of the enzyme and analysis of the inhibition by glucose-6-P. The preparation was free of glucose-6-P and 6-phosphogluconate dehydrogenases, phosphoglucomutase, and phosphofructokinase. Phosphoglucose isomerase was present in amounts varying from 5 to 15% of the hexokinase activity. ATPase accounted for 5–20% of the ADP which was formed in the pyruvate kinase–lactate dehydrogenase coupled assay of hexokinase. The hexokinase preparation retained 60–100% of its activity on storage at 2° after 1 week. Some additional purification was obtained by acetone precipitation and ammonium sulfate fractionation (9). However, this was accompanied by considerable loss in activity and alteration of the kinetics of inhibition of the dog heart enzyme (see below).

A 2.5-fold purification of the soluble heart hexokinase was obtained by ammonium sulfate fractionation with a 37–66% yield at the first and a 15–35% yield at the second precipitation. The final material did not redissolve easily and rapidly lost activity on freezing or storage at 2°. The preparation was free of interfering dehydrogenases, but contained a large amount of phosphoglucose isomerase and of ATPase activity.

The Kinetics of Inhibition by Glucose-6-P

Figure 2 illustrates the changes with time in glucose phosphorylation that occurred under conditions in which no glucose-6-P could accumulate, where such accumulation did occur, and where the inhibitor was added at the beginning of incubation. Similar results were obtained with intact mitochondria and the partly purified mitochondrial hexokinase from all species and with the partly purified enzyme from the soluble fraction of dog and guinea pig heart. Adequate measurement of the inhibited reaction was not possible with the degree of purification which was achieved for the soluble enzyme from rat and rabbit heart.

The inhibitory effect of glucose-6-P cannot be due to the occurrence of the reverse reaction because of the free energy liberated in the forward reaction and the fact that either product (ADP or glucose-6-P) alone is inhibitory (3). Furthermore, the effect of glucose-6-P is independent of glucose concentration (see below) (3). The equations for bimolecular reactions described by Reiner (24) were modified to include product inhibition of this type so as to yield the following relationship in which V_u is the velocity of the uninhibited reaction (measured in the presence of glucose-6-P dehydrogenase), V_i is the velocity of

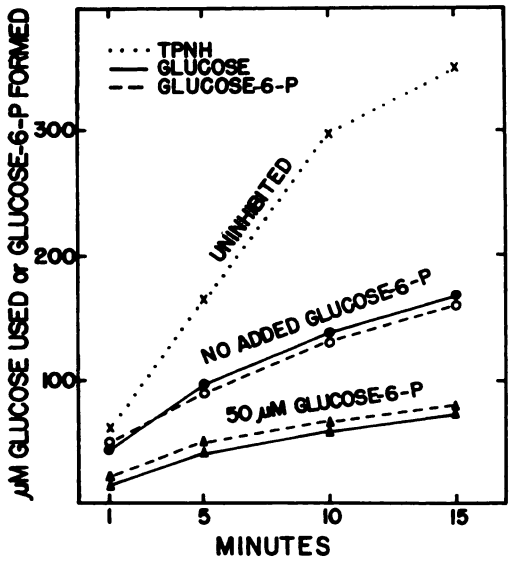

FIG. 2. *Plot of hexokinase activity with time*

The dotted line is a plot of the TPNH concentration in micromoles per liter produced when the enzyme is coupled with glucose-6-P dehydrogenase and thus represents the velocity attained in the absence of glucose-6-P. The incubation contained 1 mM glucose, 3 mM ATP, 1 mM TPN^+, and 475 μg protein of solubilized mitochondrial extract from rat heart per milliliter. Otherwise conditions are as described in Methods section. The decrease in velocity after 10 min incubation at 30° is independent of substrate concentration. The solid and dashed lines represent, respectively, the concentration of glucose used and glucose-6-P in micromoles per liter formed during incubation without TPN^+ and glucose-6-P dehydrogenase. Substrate and heart extract concentrations were as described above.

the reaction in which inhibitor accumulated or to which it was added, and γ is the slope: $V_u/V_i = 1 + \gamma$ [glucose-6-P].[4] A linear relationship was found when glucose-6-P concentration was plotted against V_u/V_i, and the ordinate was intercepted where this ratio was 1 and inhibitor concentration was 0 (Figs. 3–5). The apparent K_i of glucose-6-P was then taken as that concentration where $V_u/V_i = 2$ (Table 4).

Various preparations of hexokinase from dog heart were potently inhibited by glucose-6-P; the apparent K_i for intact, freshly prepared mitochondria was 5 $\times 10^{-5}$ M. On solubilization of the enzyme this value increased somewhat, but a much greater rise occurred when this preparation and the soluble enzyme were aged (Fig. 3,

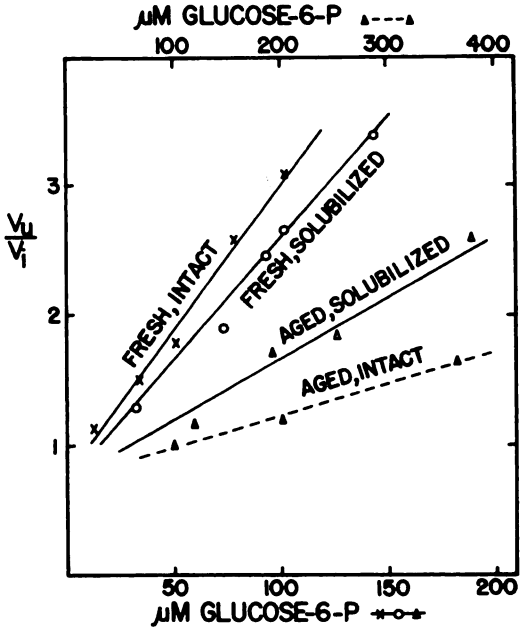

FIG. 3. *Plot of the ratio of the uninhibited hexokinase reaction velocity, V_u (coupled with glucose-6-P dehydrogenase) to the velocity determined under conditions such that glucose-6-P accumulated, V_i, against inhibitor-product concentration in micromoles per liter*

Dogs were sacrificed and mitochondria and solubilized extract therefrom were assayed within 12 hr. The aged preparations were stored at 2° for 8 days. During incubation glucose and ATP concentrations were 1 and 3 mM, respectively. Velocities were calculated as the changes in glucose (or glucose-6-P) concentrations between two consecutive points on curves plotted as in Fig. 2 or from the rate of DPN^+ formation when hexokinase was coupled with the pyruvate kinase–lactic acid dehydrogenase system from data plotted as in Fig. 2. For aged, intact mitochondria (- - - -) the scale on the ordinate is reduced.

Each point represents the average of 3–9 experiments. Curves were fitted by the method of least squares and their slopes were compared by means of the t test. All curves differ from each other with a $P < 0.01$.

[4] We wish to express our gratitude to Dr. John M. Reiner, Department of Microbiology for the derivation of this equation and for his help in interpreting our data.

TABLE 4
Kinetic characteristics of hexokinases from heart

Species	Preparation	Fresh[a]	Aged[b]	Relative recovery[c]	Glucose-6-P apparent K_i $\times 10^5$	ATP apparent K_i $\times 10^5$
Dog	Mitochondria, intact	X	—	100	5.3 ± 0.3[d] (5)[e]	—[f]
	Mitochondria, intact	—	X	59	56 ± 8.2 (3)	—[f]
	Solubilized mitochondrial	X	—	92	7.0 ± 0.3 (9)	4.9 ± 0.4 (4)
	Solubilized mitochondrial	—	X	78	15 ± 0.6 (6)	18 ± 3.2 (6)
	Soluble fraction	X	—	38	6.6 ± 0.6 (3)	—[f]
	Soluble fraction	—	X	16	15 ± 1.4 (3)	—[f]
Guinea pig	Mitochondria, intact	X	—	100	11 ± 0.8 (4)	—[f]
	Solubilized mitochondrial	X	—	89	5.6 ± 0.6 (7)	7.1 ± 0.6 (3)
	Solubilized mitochondrial	—	X	77	5.6 ± 0.2 (6)	6.8 ± 0.7 (4)
	Soluble fraction	X	—	48	6.9 ± 1.2 (3)	—[f]
Rat	Mitochondria, intact	X	—	100	13.5 ± 0.7 (10)	—[f]
	Solubilized mitochondrial	X	—	88	6.0 ± 0.3 (6)	3.8 ± 0.2 (3)
	Solubilized mitochondrial	—	X	58	7.6 ± 1.3 (3)	6.8 ± 0.6 (5)
Rabbit	Solubilized mitochondrial	X	—	90	3.2, 2.5	4.1, 8.2
	Solubilized mitochondrial	—	X	60	2.5, 2.5	7.5, 6.6

[a] Experiments were conducted within 30 hours of preparation.
[b] Preparations 5–10 days old.
[c] Recovery of enzyme activity relative to fresh crude fraction of heart from which it was prepared.
[d] Standard error of the mean.
[e] Number of experiments.
[f] ATPase activity prevented estimation of K_m.

Table 4). This change was accompanied by a loss of hexokinase activity, but selective destruction of a fraction of the enzyme highly sensitive to inhibition by glucose-6-P could only partly account for the effect of aging. A 10-fold increase in the inhibition constant occurred with only a 40% decrease in activity after 8 days of storage of intact dog heart mitochondria.

Concomitant with the decrease in sensitivity to inhibition by glucose-6-P on aging there also occurred a rise in the K_m of ATP for the solubilized mitochondrial enzyme. Further, while in fresh preparations the inhibitory effect of glucose-6-P was independent of ATP concentration (Fig. 4), in preparations aged for 2–8 days it became competitive with ATP (Fig. 5).[5] It was not possible to determine whether such an alteration in kinetics also occurred on aging of intact mitochondria and of the soluble hexokinase of dog heart, because of the presence of ATPase. Aging was without effect on the K_m of glucose. This was 2.8 $\times 10^{-5}$ M in all preparations assayed between 6 hr and 8 days after excision of the hearts.

Another effect of aging on dog heart mitochondrial hexokinase was a shift in the relationship between pH and activity (Fig. 6). The freshly prepared enzyme was most active between pH 8 and 9, and was thus similar to the hexokinases described by Crane and Sols in the soluble fraction of calf heart (8) and in liver and skeletal muscle (9). After 8 days, however, the dog heart enzyme displayed a broad range of optimal activity between pH 6.5 and 8.7 which was similar to what has been described for the purified calf heart particulate enzyme (8) and brain hexokinase (3).

Thus, storage of hexokinase solubilized

[5] The possibility that these and related observations might have been complicated by the competitive inhibition of hexokinase by ADP was eliminated by (a) coupling of hexokinase with the pyruvate kinase–lactate dehydrogenase system; (b) where this was not possible by adding ATP: creatine phosphotransferase and creatine phosphate. No ADP accumulated under these conditions.

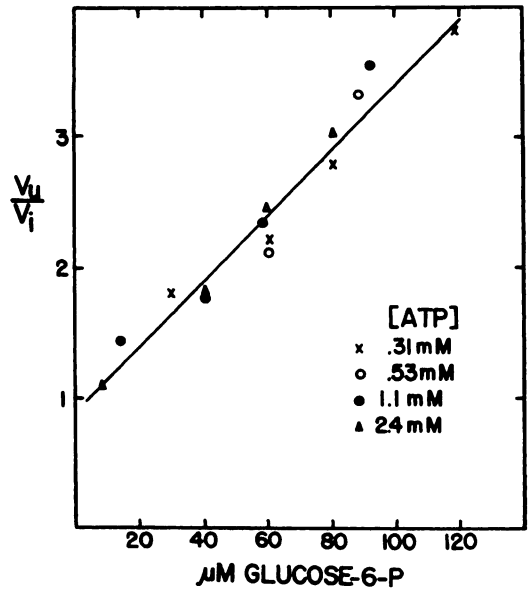

FIG. 4. *The effect of ATP concentration on the inhibition of freshly prepared solubilized dog heart hexokinase by glucose-6-P*

Each point represents the average of three experiments. The curve was fitted by the method of least squares. V_u and V_i refer to the uninhibited and inhibited reaction velocities as defined in the legend for Fig. 3.

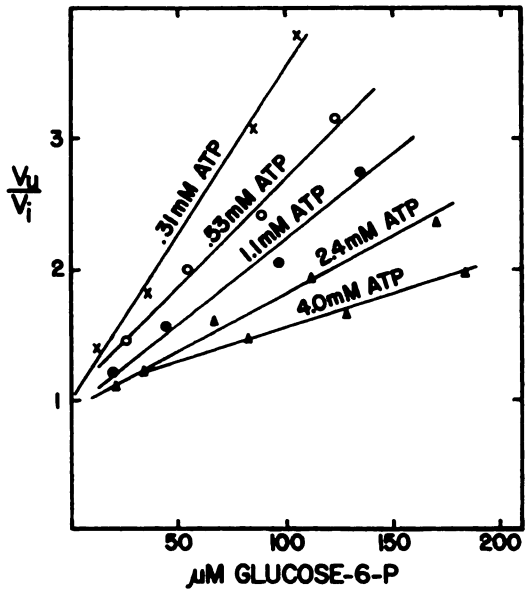

FIG. 5. *The effect of ATP concentration on the inhibition of aged solubilized dog heart hexokinase by glucose-6-P*

Each point represents the average of four experiments. The curves were fitted by the method of least squares and differ from each other with $P < 0.01$. V_u and V_i are defined in the legend for Fig. 3.

from dog heart mitochondria apparently resulted in a change in properties of the enzyme such that its affinity for the inhibitor and for one of its substrates, ATP, decreased, while concurrently the interaction between these two substances changed from a noncompetitive to a competitive one.

Such a relationship was not found on examination of mitochondrial and soluble hexokinases from guinea pig and mitochondrial enzyme from rat and rabbit heart (Table 4). In contrast to dog, solubilization of the guinea pig and rat particulate hexokinases resulted in a twofold decrease in the apparent K_i of glucose-6-P. Aging of these preparations and of guinea pig soluble enzyme did not result in any further change in the inhibitor constant although a significant drop in activity on storage was observed. In preparations from all three species the interaction between glucose-6-P and ATP remained noncompetitive. Aging of rat heart enzyme did result in a relatively small but significant rise in the K_m of ATP (Table 4). Of all the hexokinases which were examined, the one associated with rabbit heart mitochondria was most sensitive to inhibition by glucose-6-P; the inhibitor constant was 3×10^{-5} M.

Hexokinase prepared from rat brain mitochondria showed no change in sensitivity to inhibition by glucose-6-P upon aging ($K_i = 2 \times 10^{-4}$ M). Furthermore, this inhibition was found to be noncompetitive with ATP in confirmation of what had previously been observed by Crane and Sols (26) but contrary to what has been reported by Fromm and Zewe (27).

Effect of Inorganic Phosphate on Glucose-6-P Inhibition

P_i has been shown to stimulate glucose utilization in red blood cells by overcoming the effectiveness of glucose-6-P as an inhibitor of glucose phosphorylation (28). A similar effect was observed recently by

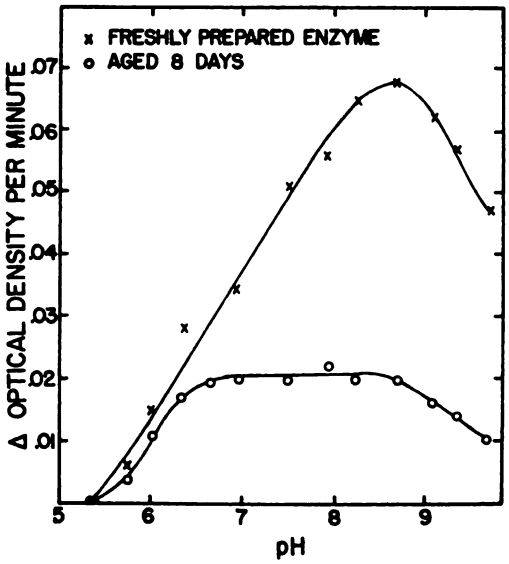

FIG. 6. *The effect of pH on hexokinase prepared from dog heart mitochondria*

The ordinate represents activity of the uninhibited reaction coupled with glucose-6-P dehydrogenase. The data are corrected for the fact that below pH 6.4 and above 8.7 TPNH was produced in the absence of glucose and ATP. Tris-maleate-NaOH buffers were prepared as described by Gomori (25). The same preparation (197 μg/ml) was used at each of the two times.

Uyeda and Racker in a system reconstructed with glycolytic enzymes including tumor hexokinase (29). The inhibition of the latter enzyme by glucose-6-P was partly reversed by P_i (30). A similar effect was observed by us on partly purified mitochondrial preparations from guinea pig and dog heart (Table 5). A 2- to 4-fold increase in the apparent K_i of glucose-6-P occurred upon addition of P_i in concentrations which are within the order of magnitude of those found within muscle fibers. Upon aging of dog heart hexokinase the decrease in effectiveness of glucose-6-P as an inhibitor was accompanied by failure of P_i to further antagonize glucose-6-P induced inhibition. No alteration in this interaction was observed on aging of the guinea pig preparation.

TABLE 5

Action of orthophosphate on glucose-6-P inhibition of heart mitochondrial hexokinases

Solubilized enzymes were prepared as described in methods. Each concentration of P_i was tested at 3 to 4 concentrations of glucose-6-P in the range of 30–300 μM.

P_i ($\times 10^3$ M)	Glucose-6-P apparent $K_i \times 10^5$ M			
	Dog		Guinea pig	
	Fresh	Aged	Fresh	Aged
0	4.5	47	5.4	5.7
1	8.9	42	9.3	10.0
3	11.8	42	13.2	14.0
10	22.5	47	26.5	25.2

DISCUSSION

The subcellular distribution of enzymes in heart muscle is not nearly as clear as that observed in tissues such as liver. The marked effect of tonicity and composition of the medium on the activity of heart sarcosomal (mitochondrial) enzymes was first described by Cleland and Slater (31). The morphology of rabbit heart mitochondria, as seen in the electron microscope, has also been shown to depend upon the medium used for isolation with sucrose producing the greatest apparent distortion (32). Finally, Siekevitz (33) has indicated that particles having both the morphological and enzymic characteristics of microsomes could be produced by repeated washing of heart mitochondria. The compartmentalization of hexokinase in myocardial fibers thus presents a problem which depends upon the medium of isolation and also on the species studied. Hernandez and Crane (34) demonstrated a strong association of hexokinase with pig heart mitochondria which could, however, be disrupted by manipulation of pH and ionic strength in the presence of salts or of glucose-6-P. These studies and the present ones suggest that a large fraction of heart hexokinase is bound, perhaps in a reversible manner, to the mitochondrial surface (35). Glucose utilization may thus be influenced by the proximity of hexokinase to other, soluble, glycolytic enzymes, by the rate of removal of the inhibitory product of the reaction, and by the availability of inorganic phosphate to reverse the in-

hibitory effect of glucose-6-P. However, it is possible that the association of hexokinase with mitochondria is an artifact of adsorption of the soluble enzyme to mitochondria (or something sedimenting with mitochondria) which occurs on homogenization of the muscle fibers.

Aging for 2–8 days resulted in marked alterations in the kinetics of inhibition of dog heart hexokinase, but not in the cardiac enzyme prepared from other species. The most notable change was in the appearance of a competitive interaction between glucose-6-P and ATP. This and associated observations suggest that a change occurred in the enzyme, so that glucose-6-P had a high affinity for a site other than the active center when the enzyme had been freshly prepared, but on aging attachment occurred only to the active center and with a decreased affinity. The observed changes can be attributed to the formation of an equilibrium between two forms of heart hexokinase. The existence of two forms has been observed in rat heart and aging was found to affect the ratio of the two forms in fat pads (36). However, the alteration in kinetics of dog heart hexokinase could also have resulted from the action of a proteolytic enzyme in the partly purified preparations from dog. Such effects should be considered when comparing the results of different investigators who have viewed the interaction of glucose-6-P and ATP as competitive (27) and noncompetitive (8, 9, 26). Furthermore, it suggests the possibility that purification and storage of an enzyme may result in changes such that the kinetic properties may no longer reflect what are presumably the characteristics of the enzyme as a functional part of the tissue.

Interpretation of the data on inhibition of heart hexokinase by glucose-6-P in terms of control of this enzyme in intact tissue requires information about the rate of glucose phosphorylation and glucose-6-P concentration in cardiac muscle. The most precise data on the rate of glucose phosphorylation have been obtained in perfused rat hearts (2, 37, 38). It is evident that factors which influence the concentration of glucose-6-P in such hearts, particularly through the regulation of phosphofructokinase, affect the rate of glucose phosphorylation. It is thus interesting to determine the degree of inhibition of glucose phosphorylation which can be attributed to inhibition of heart hexokinase by intracellular glucose-6-P (Table 6). The total hexokinase activity exceeds the maximal rate of glucose phosphorylation by a factor of 5 (rat) to 10–20 (dog). However, if one assumes that the distribution of intracellular glucose-6-P is uniform relative to the distribution of hexokinase, then our data lead to the estimate that 80–90% of the rat heart enzyme activity and 70% of that of the dog heart is inhibited under control conditions. An increase in glucose-6-P concentration could inhibit rat heart hexokinase to the point of limiting the rate of glucose phosphorylation. This was verified by the administration of a large dose of epinephrine to dogs or rats which resulted in an increase in glucose-6-P concentration, presumably a decrease in hexokinase activity, and consequent accumulation of intracellular glucose. This effect of epinephrine is probably mediated through the stimulation of glycogenolysis where phosphofructokinase remains a rate-limiting step. In the intact animal it could also involve the lipolytic effect of the amine. An increase in circulating free fatty acids and their uptake by the heart could result in additional inhibition of phosphofructokinase by citrate which has been shown to accumulate in the presence of fatty acids (7, 38). However, caution should be employed in interpreting these results as indicating a physiological role of epinephrine in the regulation of glucose utilization by heart. In the dog at least, an apparent inhibition of hexokinase activity occurred only after the administration of a very large dose of epinephrine, in excess of that required to produce a maximal inotropic response (40).

A discrepancy has been noted by Ozand *et al.* (5) between the sensitivity of hexokinase in frog muscle extract to inhibition by glucose-6-P, the concentration of this ester, and the high rate of anaerobic glucose phosphorylation. The most likely explanation of this discrepancy is the reversal of

TABLE 6

Relation between glucose phosphorylation, hexokinase activity and glucose-6-P and glucose concentrations in heart

Glucose phosphorylation rates were obtained from the data of Regen *et al.* (2) on the isolated perfused rat heart and of Danforth *et al.* (39) on the dog heart perfused *in situ*. Maximal hexokinase activities are calculated from those obtained by us in the 600 *g* supernatant fractions (Table 2); K_i values for glucose-6-P are from Table 4; intracellular glucose-6-P and glucose concentrations are from the references cited above or from our unpublished observations. The estimated hexokinase activity is derived from the plot of glucose-6-P concentration versus reaction velocity as in Fig. 3.

Conditions	Maximal glucose phosphorylation rate[b]	Maximal hexokinase activity[b]	Glucose-6-P apparent K_i (mM)[a]	Glucose-6-P concentration (mM)[a]	Glucose concentration (mM)[a]	Estimated hexokinase activity[b]
Rat, intact	—	25	0.06–0.14	0.3	0	4–5
Rat, perfused	5–6	—	—	0.3	0	—
Rat, intact, epinephrine	—	—	—	1.4	19	0.5
Dog, intact	1.1–2.5	25	0.06	0.13	0	8
Dog, epinephrine, 10 μg/kg	—	—	—	0.48	8	2.7

[a] With reference to intracellular water.

[b] Micromoles per gram min^{-1} dry weight.

glucose-6-P-induced inhibition by inorganic phosphate; a rise in the concentration of the latter occurred anaerobically. Regen *et al.* (2) observed a 35% increase in glucose phosphorylation when the perfused rat heart was shifted from an aerobic to an anoxic environment with a rise in inorganic phosphate from 4 to 13 mM. We observed a greater than 2-fold increase in the apparent K_i of glucose-6-P when dog or guinea pig heart mitochondrial hexokinase was incubated with similar concentrations of phosphate. It remains to be determined whether the dissociation between glucose-6-P concentration and glucose phosphorylation which has been observed in perfused rat hearts (41, 42) is due to the action of inorganic phosphate.

Thus regulation of heart hexokinase can be correlated with the kinetics of this enzyme. However, the physiological significance of this information will require considerably more evaluation. The inhibition of hexokinase is a linear function of glucose-6-P concentration. While the large changes in concentration of this ester necessary to alter glucose phosphorylation significantly can be observed in anoxia or after a very large dose of epinephrine (Table 6), the regulation of glucose phosphorylation under normal conditions may involve more subtle factors.

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