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Effect of Perfusion with Different Substrates and with Isoproterenol on Phosphofructokinase Activity in the Isolated Guinea Pig Heart

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SUMMARY

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Guinea pig hearts were perfused with Krebs-bicarbonate solution to which glucose, pyruvate, acetate, β-hydroxybutyrate, or octanoate was added. Heart extracts were prepared in the presence of 13 mM caffeine and assayed for phosphofructokinase activity. When assays were done at pH 6.9 (a pH which allows demonstration of allosteric regulation), extracts from glucose-perfused hearts had high enzyme activity, while extracts from pyruvate-, β-hydroxybutyrate-, and octanoate-perfused hearts had very low activity. Extracts from acetate-perfused hearts had intermediate activity. The reduction in phosphofructokinase activity in pyruvate-perfused hearts was correlated with increased tissue citrate levels. Furthermore, when enzyme activity was determined as a function of fructose-6-P concentration, a sigmoid curve was obtained for extracts of pyruvate-perfused hearts and a hyperbolic curve was obtained for extracts of glucose-perfused hearts. Addition of isoproterenol to the perfusate antagonized the effect of pyruvate. The data provide direct evidence for allosteric control of the enzyme in intact tissue by intermediary metabolites of aerobic metabolism.

INTRODUCTION

Most of our knowledge regarding the activity of phosphofructokinase in response to changes in cellular metabolism has been based on estimates of substrate levels in tissues (1–3). A decrease in the concentration of fructose-6-P and an increase in that of fructose-1,6-P_2 in the tissue are consistent with an increase in phosphofructokinase activity. Recently we reported more direct evidence of a change in phosphofrutokinase activity of rabbit skeletal muscle following intravenous administration of epinephrine (4). Muscle extracts were prepared in the presence of caffeine and assayed under conditions optimal for allosteric kinetics (pH 6.9 and low fructose-6-P concentration) and in the presence of cyclic AMP. Enzyme activity in extracts of muscle samples prepared following epinephrine

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administration was much higher than activity in control extracts (4). On the other hand, maximal enzyme activity (assayed at pH 8.2) was not significantly changed. The increase in phosphofructokinase activity following epinephrine administration appeared to be due to a modification in the kinetic properties of the enzyme: it became less sensitive to ATP inhibition and had a higher affinity for fructose-6-P. The present study was undertaken to test the effect of perfusing the guinea pig heart with different substrates and with isoproterenol on phosphofructokinase activity. The results indicate that phosphofructokinase activity, when assayed at pH 6.9, could be inhibited following perfusion of the heart with pyruvate, β-hydroxybutyrate, octanoate, or acetate. Inhibition of phosphofructokinase by perfusion with pyruvate was antagonized by the addition of isoproterenol to the perfusion fluid.

MATERIALS AND METHODS

Perfusion of guinea pig hearts. Male guinea pigs (400–600 g) were decapitated; their hearts were quickly removed and transferred to a Petri dish containing perfusion fluid and were trimmed of extraneous tissue. Hearts were perfused by Langendorf's (5) technique. The perfusion fluid was Krebs-bicarbonate solution which contained 118.5 mM NaCl, 4.75 mM KCl, 2.54 mM $CaCl_2$, 1.18 mM KH_2PO_4, 1.18 mM $MgSO_4$, and 24.9 mM $NaHCO_3$ and was gassed with 95% O_2–5% CO_2. Drugs and substrates were added to this fluid as indicated. The temperature was 37°, and perfusion pressure was 60 cm H_2O. After perfusion for 1 hr heart samples were taken for assays.

Preparation of heart extracts. All procedures were carried out at 0–4°. Tissues were homogenized with all-glass Potter-Elvehjem homogenizers.

1. Extracts for phosphofructokinase assays: The apex of the heart was quickly removed, blotted, and frozen with Wollenberger clamps (6) which had been chilled in Dry Ice. The resulting frozen wafer was weighed and homogenized in 10 volumes of 10 mM glycylglycine buffer (pH 7.5), 5 mM EDTA, 20 mM NaF, and 13 mM caffeine. The homogenate was centrifuged at 17,000 × *g* for 15 min. The resulting supernatant fluid was used for phosphofructokinase assays.

2. Extracts for citrate determinations: The lower two-thirds of the ventricles were quickly removed, blotted, and frozen with chilled Wollenberger clamps. The resulting frozen samples were weighed and homogenized in 2 volumes of 0.6 M $HClO_4$. The homogenate was centrifuged at 17,000 × *g* for 15 min, and the pellet was discarded. The supernatant fluid was brought to pH 7.6 with 1 M K_2CO_3; the resulting $KClO_4$ was removed by centrifugation at 17,000 × *g* for 15 min. Citrate content was determined in this second supernatant fraction.

Assays. Phosphofructokinase was assayed spectrophotometrically by coupling the enzyme with aldolase, triosephosphate isomerase, and α-glycerol 1-phosphate dehydrogenase as described previously (4). For assays at pH 6.9 the reaction mixture contained 50 mM imidazole buffer (pH 6.9), 0.01% albumin, 0.05 mM ATP, 0.0425 mM cyclic 3′,5′-AMP, 0.102 mM NADH, 12.5 mM cysteine, 0.50 mM $MgCl_2$, 0.25 mM fructose-6-P, and coupling enzymes in a volume of 0.50 ml. The pH was adjusted to 6.9 with KOH. For assays at pH 8.2 the reaction mixture contained 50 mM potassium glycylglycine, 0.01% albumin, 0.102 mM NADH, 12.5 mM cysteine, 0.50 mM ATP, 1.0 mM $MgCl_2$, 1.0 mM fructose-6-P, and coupling enzymes. It was necessary to adjust the amount of coupling enzymes to be used at pH 6.9 because of the variation of aldolase activity in different heart extracts. Concentrations of aldolase substantially above that necessary to couple the two reactions inhibited phosphofructokinase. Such inhibition appeared to be due to the removal of fructose-1,6-P_2 from the allosteric sites of phosphofructokinase (7). The concentration of aldolase added ranged from 0.032 to 0.160 unit/ml of reaction mixture. The reaction was started by the addition of 10 μl of heart extract. Values for enzyme activity were corrected for nonspecific NADH oxidation by subtracting absorbance changes observed in reaction mixtures from which substrates were omitted. Enzyme activity is reported either as units per gram, wet weight, or as a ratio of the activity at

pH 6.9 or pH 7.3 to that at pH 8.2. These ratios were considered most suitable for this investigation because they provide an index of the proportion of phosphofructokinase active near neutrality relative to total enzyme activity (assayed at pH 8.2). In addition the assay at pH 8.2 provides a check against changes in enzyme activity due to denaturation or variation of enzyme activity from one animal to another. A unit of phosphofructokinase is the amount of enzyme that catalyzes the formation of 1 μmole of fructose-1,6-P_2 per minute.

Citrate was assayed by a modification of the method of Moellering and Gruber (8). Fresh citrate assay mixture was made each day by adding 9.95 ml of 0.10 M triethanolamine buffer, pH 7.6, and 0.05 ml of 30 mM $ZnCl_2$ to a vial which contained 1 mg (1.28 μmoles) of NADH. The assay was carried out in a spectrophotometer cuvette which contained 700 μl of citrate assay mixture, 290 μl of heart extract, and 5 μl of malate dehydrogenase (27.5 units). Initial absorbance at 340 nm was measured on a Zeiss PMQ spectrophotometer. Citrate lyase (5 μl, 0.2 unit) was added, and the reaction was allowed to reach equilibrium. The difference between initial and final absorbance was determined, and the concentration of citrate was calculated from a standard curve.

Materials. The materials used in these experiments were obtained from the following sources: aldolase, triosephosphate isomerase, α-glycerol-1-phosphate dehydrogenase, citrate lyase, and malate dehydrogenase, Boehringer; ATP, NADH, isoproterenol hydrochloride, potassium pyruvate, and octanoic acid, Sigma; sodium β-hydroxybutyrate, Calbiochem; albumin, Armour; glucose and sodium acetate, Baker. All standard laboratory chemicals were reagent grade from various sources, except for triethanolamine, which was purified by distillation before use.

RESULTS

Effect of perfusion with various substrates on phosphofructokinase activity. Phosphofructokinase activity was measured at both pH 6.9 and pH 8.2 in heart extracts following perfusion either without substrates or with various substrates added to the perfusion fluid. The results summarized in Table 1 show that enzyme activity at pH 8.2 did not change to a significant degree following perfusion with different substrates. Significant changes were observed, however, when the enzyme activity was measured at pH 6.9. For example, pyruvate at a concentration of 3.6 mM in the perfusion fluid caused a marked reduction of enzyme activity at pH 6.9 when compared with the activity of extracts from hearts perfused with glucose or with no added substrate. Thus the ratio between enzyme activity at pH 6.9 and at pH 8.2 was reduced from 0.29 without substrate to 0.01 when the hearts were perfused with pyruvate. The relationship between pyruvate concentration in the perfusion fluid and phosphofructokinase activity is summarized in Fig. 1. In these experiments the pH 6.9:8.2 phosphofructokinase activity ratio is plotted as a function of pyruvate concentration. Phosphofructokinase activity at pH 8.2 was not significantly changed, and therefore these ratios signify only changes at pH 6.9. Figure 1 shows a close relationship between pyruvate concentration in the perfusion fluid and the decrease in enzyme activity at pH 6.9. At pyruvate concentrations greater than 2.4 mM, the pH 6.9:8.2 activity ratio was virtually zero.

Following perfusion with glucose, enzyme activity at both pH 6.9 and pH 8.2 (Table 1) was not significantly different from that following perfusion with no substrate when endogenous glycogen would have been a source of energy. Perfusion of the hearts with β-hydroxybutyrate or octanoate, as in the case of pyruvate, resulted in no change in enzyme activity at pH 8.2 and virtually no activity at pH 6.9. The effect of perfusion with acetate was tested on eight separate hearts at concentrations from 1.2 to 7.2 mM. While the activity at pH 8.2 following perfusion was not significantly changed, the activity at pH 6.9 was reduced but not to the same extent as with pyruvate and related substrates. Acetate at concentrations ranging from 2.5 to 7.2 mM in the perfusion fluid reduced the pH 6.9:8.2 activity ratio to 0.15. These experiments indicate that when the heart was dependent

TABLE 1

Phosphofructokinase activity in extracts from hearts perfused with different substrates

Guinea pig hearts were perfused with Krebs-bicarbonate solution which contained the indicated substrates. Preparation of heart extracts and conditions for enzyme assays at pH 6.9 and at pH 8.2, as well as other experimental procedures, are described under MATERIALS AND METHODS. Results are expressed as units per gram, wet weight. When the number of experiments was three or more the standard deviation is shown, and when the number of experiments was two the range is given.

Substrate	Concentration	No. of experiments	Phosphofructokinase activity		
			pH 6.9	pH 8.2	pH 6.9:8.2
	mM		*units/g (wet wt)*		
None		4	2.8 ± 0.4	9.8 ± 2.0	0.29
Glucose	10.0	2	4.4 (3.8–5.4)	12.0 (11.0–13.0)	0.37
	4.8	1	6.6	17.6	0.38
Pyruvate[a]	3.6	3	0.4 ± 0.2	10.2 ± 2.2	0.03
β-Hydroxybutyrate	4.8	2	0	17.0 (16.0–18.0)	0
Octanoate	1.3	2	0	14.0 (13.8–14.2)	0
	1.8	2	0	15.2 (14.6–15.8)	0
Acetate	2.0	1	2.4	14.4	0.17
	2.8	1	1.8	11.8	0.15
	3.6	1	2.4	12.6	0.19
	4.0	1	2.0	14.0	0.14
	6.0	1	1.4	10.8	0.13
	7.2	1	2.0	12.0	0.17

For complete data on pyruvate, see Fig. 1.

on glucose or glycogen, phosphofructokinase activity was high at pH 6.9. On the other hand, perfusion with substrates which are oxidized through the Krebs cycle without the participation of phosphofructokinase resulted in low enzyme activity at pH 6.9.

Effect of isoproterenol on phosphofructokinase activity. It is well accepted that the catecholamines can increase cardiac contractility (9) and can also stimulate glycogenolysis through activation of glycogen phosphorylase. If the Embden-Meyerhof pathway is to contribute to energy production to support such an increase in cardiac contractility, phosphofructokinase must also be activated. The question then arose whether inhibition of the enzyme by perfusion with pyruvate could be antagonized by isoproterenol. The results summarized in Fig. 1 illustrate the relationship between pyruvate concentrations and pH 6.9:8.2 activity ratios when the perfusion fluid contained 4 μM isoproterenol. Perfusion with the catecholamine did not cause any significant change in the activity at pH 8.2. On the other hand, the presence of isoproterenol in the perfusion fluid antagonized phosphofructokinase inhibition due to perfusion with pyruvate. For example, at 1.35 mM pyruvate the pH 6.9:8.2 activity ratio was 0.07, while in the presence of the catecholamine and the same pyruvate concentration the ratio was increased to 0.35. The presence of pyruvate at concentrations above 1.35 mM in the perfusion fluid caused a marked reduction in enzyme activity at pH 6.9 even in the presence of isoproterenol (Fig. 1). The specificity of the catecholamine effect on phosphofructokinase was examined by replacing the *beta* receptor agonist, isoproterenol, with the *alpha* receptor agonist, phenylephrine. Perfusion with phenylephrine, even at a concentration of 400 μM (100 times the concentration of isoproterenol used), produced no detectable change in enzyme activity.

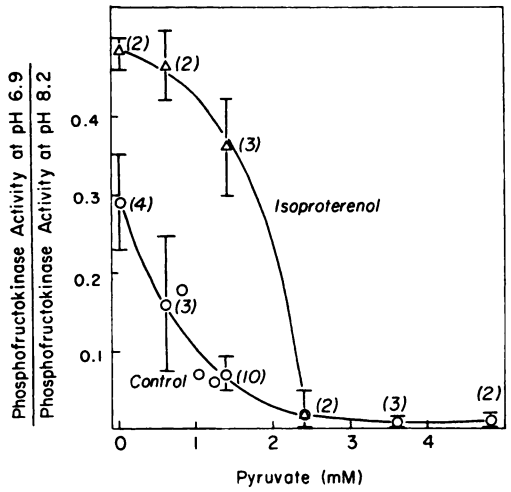

FIG. 1. *Phosphofructokinase activity of extracts from hearts perfused with pyruvate and isoproterenol*

Guinea pig hearts were perfused with Krebs-bicarbonate solution containing the indicated concentrations of pyruvate. Perfusion fluid for the control (O——O) contained no isoproterenol, while experiments carried out for the isoproterenol curve (△——△) contained a 4 μM concentration of the catecholamine. Enzyme activity is expressed as the ratio between activity at pH 6.9 and activity at pH 8.2. Each point represents the value obtained from one heart or the mean of values obtained from the number of hearts indicated in parentheses; the vertical bars indicate the standard deviation or the range when only two values were obtained. Other experimental conditions are described under MATERIALS AND METHODS.

Citrate levels in hearts following perfusion with different substrates. The results reported above show that perfusion of hearts with different substrates influences phosphofructokinase activity in heart extracts. An attempt was subsequently made to determine the mechanism of such inhibition. A previous report from this laboratory strongly suggested a ligand-mediated modification of the enzyme (4). It is well known that ATP and citrate inhibit phosphofructokinase, and it has been postulated that both may serve to regulate the enzyme *in vivo* [for references, see review (10)]. Experiments performed to determine ATP levels in hearts perfused with various levels of pyruvate and glucose showed, in agreement with the results reported earlier by Williamson (11), that the levels of the ATP did not vary appreciably. In contrast, tissue levels of citrate were increased markedly following perfusion of the heart with pyruvate (Fig. 2). For example, the citrate content of hearts perfused with 4.8 mM pyruvate was increased approximately 22-fold above the levels in hearts perfused without pyruvate. Figure 2 also shows that addition of isoproterenol to the pyruvate perfusion fluid resulted in a reduction in citrate levels. Comparison of the results presented in Fig. 2 with those in Fig. 1 reveals that perfusion conditions which led to low enzyme activity at pH 6.9 also resulted in high levels of citrate in the heart.

Changes in allosteric kinetics of phosphofructokinase following perfusion with different substrates. The data presented in Table 1 and Fig. 1 show that significant changes in phosphofructokinase activity which result from perfusion with different substrates can only be demonstrated at pH 6.9 and not at

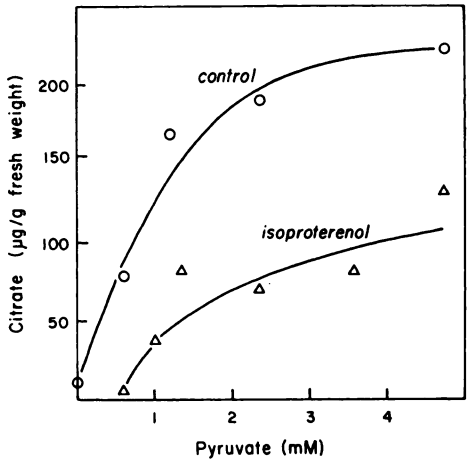

FIG. 2. *Citrate content of hearts perfused with pyruvate or with pyruvate and isoproterenol*

Guinea pig hearts were perfused with Krebs-bicarbonate solution containing the pyruvate concentrations indicated on the abscissa. The perfusion fluid contained either no isoproterenol (O——O) or a 4 μM concentration of the catecholamine (△——△). Each value was obtained from one heart. Other experimental conditions, including the determination of citrate, are described under MATERIALS AND METHODS.

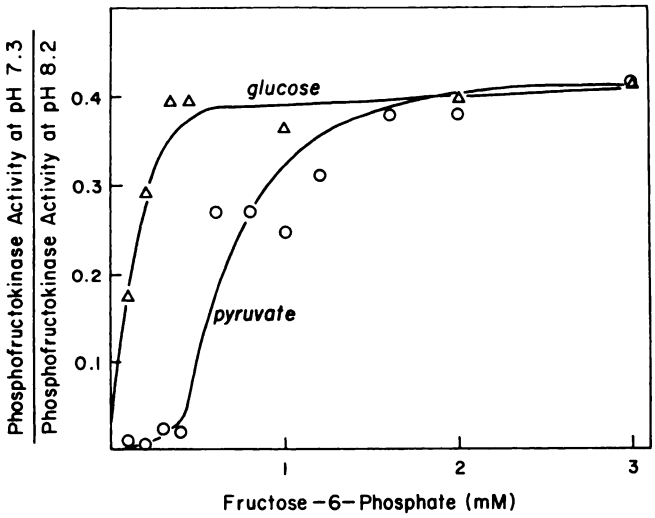

FIG. 3. *Effect of fructose-6-P concentration on phosphofructokinase activity in extracts from hearts perfused with either glucose or pyruvate*

Guinea pig hearts were perfused with Krebs-bicarbonate solution containing either 4.8 mM glucose (△——△) or 4.8 mM pyruvate (○——○). Heart extracts were prepared following perfusion for 1 hr, and phosphofructokinase activity was assayed at pH 8.2 as described under MATERIALS AND METHODS. Assays at pH 7.3 were carried out in a reaction mixture identical with that described in Table 2 except that the concentration of ATP was fixed at 0.45 mM and the concentration of fructose-6-P was varied from 0.1 to 3 mM. Other experimental conditions are described under MATERIALS AND METHODS.

pH 8.2. These findings coincide with the fact that allosteric kinetics of the enzyme can be observed at pH 6.9 and not at pH 8.2. The hypothesis that these changes in enzyme activity may be due to a change in the allosteric kinetics of the enzyme was examined. Kinetics of the enzyme was tested at pH 7.3 rather than pH 6.9. This pH was preferred because the enzyme is highly sensitive to ATP inhibition at pH 6.9. Moreover, titration curves for fructose-6-P were obtained more easily at pH 7.3 than at pH 6.9. The results illustrated in Fig. 3 show that the affinity of phosphofructokinase for fructose-6-P at pH 7.3 was reduced in extracts from hearts perfused with pyruvate when compared to extracts from glucose-perfused hearts. The apparent K_m values were 0.65 mM and 0.11 mM, respectively. Figure 4 shows a titration curve for fructose-6-P of phosphofructokinase activity in extracts from hearts perfused with pyruvate, compared with that from hearts perfused with pyruvate and isoproterenol. The results show that when the catecholamine was included in the perfusion fluid the K_m for fructose-6-P was

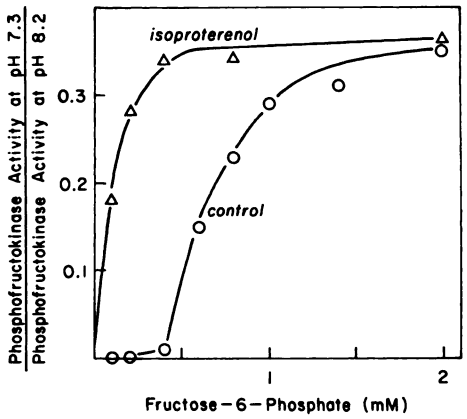

FIG. 4. *Effect of isoproterenol on apparent K_m for fructose-6-P of phosphofructokinase in extracts from hearts perfused with pyruvate*

Guinea pig hearts were perfused with Krebs-bicarbonate solution containing 1.2 mM pyruvate either without (○——○) or with 4 μM isoproterenol (△——△). Enzyme assays and other experimental conditions were the same as in Fig. 3.

significantly reduced and the shape of the titration curve was changed from sigmoid to hyperbolic.

Phosphofructokinase in extracts from

TABLE 2

Effect of perfusion of guinea pig hearts with pyruvate on sensitivity of phosphofructokinase to inhibition by ATP

Guinea pig hearts were perfused with Krebs-bicarbonate solution containing either 4.75 mM glucose or 4.75 mM pyruvate. Heart extracts were prepared following perfusion for 1 hr, and phosphofructokinase activity was assayed both at pH 7.3 and at pH 8.2. Assays at pH 7.3 were carried out in a reaction mixture identical with that described under MATERIALS AND METHODS for assays at pH 6.9, except that the imidazole buffer was at pH 7.3 and cyclic AMP was not included. In the assays at pH 7.3 the fructose-6-P concentration was fixed at 0.8 mM and the ATP concentration was varied from 0.012 to 0.143 mM. Other exoerimental conditions for preparation of extracts and for assay at pH 8.2 are described under MATERIALS AND METHODS. Enzyme activity at pH 8.2 in experiment 1 was 18.4 units/g following perfusion with glucose and 13.8 units/g following perfusion with pyruvate, and in experiment 2 it was 14.4 and 9.8 units/g, respectively.

Expt.	ATP	Phosphofructokinase activity			
		Glucose perfusion		Pyruvate perfusion	
		pH 7.3 activity	pH 7.3:8.2	pH 7.3 activity	pH 7.3:8.2
	mM	*units/g (wet wt)*		*units/g (wet wt)*	
1	0.006			1.90	0.14
	0.012	4.26	0.23	2.78	0.20
	0.036	7.46	0.40	4.10	0.30
	0.072	6.92	0.37	0.86	0.06
	0.143	6.66	0.36	0.54	0.04
2	0.006			0.70	0.07
	0.012			1.28	0.13
	0.014	2.66	0.18	1.18	0.12
	0.036	4.42	0.31	1.18	0.12
	0.072	6.38	0.44	0.32	0.03
	0.143	6.12	0.43		

hearts perfused with pyruvate was shown to be more sensitive to ATP inhibition than that from hearts perfused with glucose. The results summarized in Table 2 show that although ATP at concentrations ranging from 0.073 to 0.143 mM caused no inhibition of the enzyme in glucose-perfused heart extracts, marked inhibition of the enzyme from pyruvate-perfused hearts was observed.

DISCUSSION

The experiments reported in this paper indicate that at pH 6.9 phosphofructokinase activity of the heart was markedly reduced following perfusion with substrates that can be oxidized through the Krebs cycle without involvement of phosphofructokinase. In contrast, total phosphofructokinase activity was not significantly affected. Of this group of substrates, pyruvate, β-hydroxybutyrate, and octanoate were the most potent. Acetate, on the other hand, failed to cause complete inhibition even at considerably higher concentrations. These effects on phosphofructokinase could possess some physiological importance as a mechanism to conserve glycogen when ample supplies of fatty acids and pyruvate are available to the heart. Thus the availability of energy from aerobic metabolism lessens the demand for energy derived from glycolysis. Previous investigations of metabolism in the perfused mammalian heart showed that glucose oxidation was decreased when fatty acids were made available to the organ (12). For example, perfusion of the heart with acetate or pyruvate was reported to reduce glucose oxidation by 80% and to cause a concomitant decrease in glycogen utilization (11). A similar effect was reported following perfusion of the rat heart with β-hydroxybutyrate or octanoate (12).

The finding that perfusion of the heart with acetate resulted in only partial inhibition of phosphofructokinase may be interpreted in the light of results reported by Randle *et al.* (13) and Williamson (11). In these experiments perfusion of the rat heart with acetate caused a decrease in glycolysis and a concomitant increase in the levels of citrate and AMP (13). The fact that citrate is a phosphofructokinase inhibitor while AMP is an activator may explain partial inhibition of the enzyme following perfusion with acetate. Perfusion with pyruvate, on the other hand, although increasing the level of citrate (see above), did not appear to affect the levels of AMP in the heart (11).

The changes in phosphofructokinase activity which occurred at a pH near neutrality and not at pH 8.2 suggest an effect on the tertiary structure of the enzyme.

Conformational changes of the enzyme are known to occur at neutral pH and not at pH 8.2. The changes reported above appear to be analogous to those we reported previously on activation of skeletal muscle phosphofructokinase following epinephrine administration (4). The effect in both cases could be explained on the basis of enzyme modification by different substrates (4). Incubation of purified phosphofructokinase with different substrates was reported previously to result in a change in the kinetics of the enzyme at pH 7.3. The results summarized in Table 2 and Figs. 3 and 4 show an increase in the K_m for fructose-6-P as well as an increase in the sensitivity of the enzyme to ATP inhibition following perfusion with pyruvate. Both effects point to a change in the allosteric kinetics of phosphofructokinase.

Results from hearts perfused with pyruvate showed that addition of isoproterenol to the perfusion fluid antagonized the pyruvate-induced inhibition of phosphofructokinase. Enzyme "activation" by isoproterenol was shown also to reverse kinetic changes caused by perfusion with pyruvate. Thus the catecholamine caused a reduction in the apparent K_m for fructose-6-P. These effects are similar to those we reported before on skeletal muscle phosphofructokinase following epinephrine administration (4). We proposed that the effect of the catecholamine on skeletal muscle enzyme was due to modification of the enzyme to a form that is more active under our assay conditions. Such modification appears to be mediated through a combination of hexose phosphates (fructose-6-P, fructose-1,6-P_2) and adenylate nucleotides (AMP, ADP) (4); the levels of these substances in the heart were reported to be increased by epinephrine (3). A similar explanation for the effect of isoproterenol on the heart enzyme can also be postulated.

It is recognized here that perfusion with pyruvate and other substrates could result in a considerable change in the intracellular levels of different cellular metabolites. It is noteworthy that an increase in citrate level in the heart was found to coincide with a decrease in enzyme activity at pH 6.9 following perfusion with pyruvate. These results suggest that the substrate responsible for tertiary modification of phosphofructokinase is citrate. Further support for such a hypothesis is the close relationship between activation of phosphofructokinase following perfusion with isoproterenol and the decrease in heart citrate levels. Such an interpretation is consistent with previous evidence that citrate is a regulator of phosphofructokinase (14, 15).

In the experiments described above as well as in those we reported before in connection with the effect of epinephrine on skeletal muscle enzyme (4), it was essential to prepare muscle extracts in the presence of caffeine in order to show the differences in phosphofructokinase activity between control and experimental samples. We postulated (4) that the purine derivative, like ATP, binds to the allosteric sites when they are not occupied by one of the activators, and consequently inhibits the enzyme. Such a hypothesis is supported by the results reported by Kemp and Krebs (16), who showed that caffeine competes with cyclic AMP for the allosteric sites on phosphofructokinase. We assume that caffeine does not bind to phosphofructokinase, which has activators such as cyclic AMP and the hexose phosphates bound to these sites. Thus more of the phosphofructokinase from hearts perfused with glucose or dependent on endogenous glycogen was present in the active conformation, i.e., with activators attached to allosteric sites; this form was protected against inhibition by caffeine during homogenization. On the other hand, enzyme in extracts from hearts perfused with pyruvate or related substrates was in the inhibited conformation, i.e., with ATP or citrate attached to allosteric sites. The enzyme in the first form following preparation of the heart extracts with caffeine will appear more active than the latter under assay conditions which are optimal for allosteric kinetics. This interpretation is supported by our previous experiments (4) on the modification of purified phosphofructokinase following incubation with various effector metabolites. For example, incubation of the pure enzyme with cyclic AMP,5′-AMP, fructose-6-P, or fructose-

1,6-P_2 resulted in modification of the enzyme to a form that was less sensitive to inhibition by caffeine and by ATP than the control enzyme. Modification of phosphofructokinase under these conditions was observed even though the enzyme during the assay was in an environment that does not have the metabolite modifier.

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