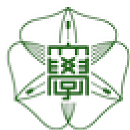

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Properties of Hepatic Hexose 6-Phosphate Dehydrogenase and Glucose 6-Phosphate Dehydrogenase from Fishes and Amphibians

By

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(*With 8 Text-figures and 6 Tables*)

Introduction

Hexose 6-phosphate dehydrogenase (H6PD) can be distinguished from glucose 6-phosphate dehydrogenase (G6PD) by its broad substrate specificity. H6PD is active on glucose 6-phosphate (G6P), galactose 6-phosphate (Gal6P), 2-deoxyglucose 6-phosphate (dG6P) and glucose in the presence of either NADP or NAD, while G6PD is relatively specific to G6P and NADP (Ohno *et al.* 1966, Shaw 1966, Beutler and Morrison 1967).

It has been predicted that G6PD and H6PD are derived from a common ancestral molecule and that the divergence occurred at the time of vertebrate emergence (Stegeman and Goldberg 1971, Yamauchi and Goldberg 1973, Stegeman and Yamauchi 1975). Recently, we have found however that the enzyme which has properties quite similar to vertebrate H6PD is present in various species of echinoderms. This suggests a possibility that the divergence of H6PD might have occurred at the time of echinoderm emergence (Mochizuki and Hori 1973, 1976, Hori *et al.* 1975).

In order to discuss the origin of H6PD, however, it is necessary to obtain more precise information on the phylogenetic distribution and the properties of H6PD and G6PD from a wide variety of animals. As the first phase of this project, this paper describes the properties of H6PD and G6PD from five species of fishes and amphibians.

Materials and Methods

Adult toads (*Bufo bufo*) were obtained from a commercial source. Adult frogs (*Rana chensinensis*), crucian carps (*Carassius carassius*) and lampreys (*Entosphenus japonicus*) were collected in the suburb of Sapporo and rockfish (*Sebastes taczanowskii*) in Ishikari Bay.

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G6P, Gal6P, dG6P, nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) were obtained from Sigma Chemical Company and DEAE-Sephadex (A-50), CM-Sephadex (C-50) and Sephadex G-200 superfine from Pharmacia. The other chemicals used were the highest grade available.

Enzyme and protein assays, molecular weight estimation and examinations of the effects of *p*-chloromercuribenzoate (PCMB), dehydroepiandrosterone (DEA) and $MgCl_2$ on enzyme activity were carried out by the methods described previously (Hori and Sado 1974). The specific activity of enzyme was expressed as units (pH 10.0) per mg protein.

Enzyme separation

All procedures were performed at 4°C. The buffer used was 20 mM phosphate buffer (pH 6.4) containing 2.5 mM ethylenediaminetetraacetate (EDTA).

All the precipitates obtained by salting out with ammonium sulfate were collected by centrifugation, dialyzed against the buffer and centrifuged to remove insoluble materials before applying on ion exchange gels which had been equilibrated with the buffer. The sizes of ion exchange columns were 2.2×20 cm (No. 1), 2.2×30 cm (No. 2), 3.3×10 cm (No. 3), 3.3×20 cm (No. 4) and 3.3×40 cm (No. 5). Washing and elution of the column was made at a flow rate of 30 ml per hour and 10 ml fractions were collected. Concentration of the enzyme solution by ultrafiltration was performed with a Diaflo XM-50 membrane (Amicon Corporation).

Toad G6PD Livers, 20 g, stored at -70°C were homogenized in 5 volumes of the buffer and centrifuged at 10,000 ×g for 10 min. The supernatant was treated with ammonium sulfate and the 20%-30% (w/v) precipitate was placed on a DEAE-Sephadex column, No. 3. The enzyme was eluted by a linear gradient made with 200 ml portions of the buffer with and without 1.0 M NaCl. Fractions with high G6PD activity were pooled and dialyzed against the buffer. The specific activity of this solution was 0.01.

Toad H6PD The supernatant obtained after separation of G6PD was brought to 35% (w/v) in respect to ammonium sulfate. The precipitate was placed on a CM-Sephadex column, No. 4, and the enzyme was eluted by a linear gradient made with 300 ml portions of the buffer and of 0.1 M Na_2HPO_4. H6PD was eluted at about pH 6.8. Fractions of H6PD activity were dialyzed and concentrated by ultrafiltration. The specific activity of this solution was 0.11.

Frog G6PD Fresh livers, 20 g, were rinsed with 0.25 M sucrose, homogenized in 9 volumes of sucrose and centrifuged successively at 750 ×g for 10 min., at 8,500 ×g for 15 min., and at 105,000 ×g for 1 hour in order to remove particle fractions. The soluble fraction was treated with ammonium sulfate and the 25%-30% (w/v) precipitate was placed on a DEAE-Sephadex column, No. 4. The enzyme was

eluted by a linear gradient made with 300 ml portions of the buffer with and without 1.0 M NaCl. Fractions of high G6PD activity were concentrated by ultrafiltration and placed on a CM-Sephadex column, No. 1. The enzyme was eluted by a linear gradient made with 200 ml portions of the buffer and of 0.1 M Na_2HPO_4. Fractions of G6PD activity were concentrated and dialyzed by ultrafiltration. The specific activity of this solution was 1.8.

Crucian carp G6PD Fresh livers, 170 g, were rinsed with 0.25 M sucrose, homogenized in 6 volumes of sucrose and centrifuged successively at 750 $\times$g for 10 min. and at 8,500 $\times$g for 15 min. The resultant supernatant was then centrifuged at 105,000 $\times$g for 1 hour to obtain cytosol and microsomal fractions.

The cytosol fraction was treated with ammonium sulfate and the 25%–35% (w/v) fraction was placed on a CM-Sephadex column, No. 5. The enzyme was eluted by a linear gradient made with 400 ml portions of the buffer and of 0.1 M Na_2HPO_4. Fractions of high G6PD activity were concentrated by ammonium sulfate (25%–35%), and placed on a DEAE-Sephadex column, No. 1. The enzyme was eluted by a linear gradient made with 150 ml portions of the buffer with and without 0.7 M NaCl. Fractions with high G6PD activity were concentrated by a collodion bag and placed on a Sephadex G–200 column (3.3$\times$45 cm). The enzyme was eluted with 20 mM phosphate buffer (pH 6.4) containing 0.1 M KCl and 2.5 mM EDTA at a flow rate of 3.7 ml per hour. One of the 5 ml fractions with the highest G6PD activity (specific activity, 237) was used in the following study (Table 1 and Fig. 1).

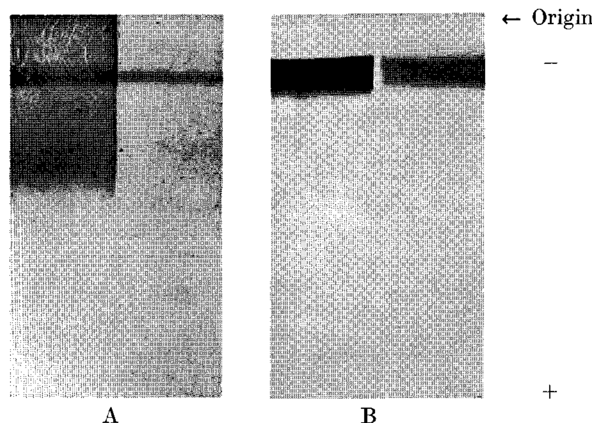


Fig. 1. Electrophoresis of purified G6PD and H6PD from crucian carp liver on polyacrylamide gel. A, G6PD; B, H6PD. In each case the strip on the left was stained for glucose 6-phosphate dehydrogenase activity and the strip on the right was stained for protein. Two bands of G6PD activity in A would probably represent the tetramer (slow) and dimer (fast).

Table 1. Purification of G6PD from crucian carp liver. Activity was assayed at pH 10.0 with G6P and NADP.

Step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)
Crude extract	1, 020	2, 450*	9, 180	0. 267	100
Cytosol fraction	950	2, 190*	7, 980	0. 274	89
Ammonium sulfate	83	1, 310	1, 220	1. 07	53
CM-Sephadex	100	770	128	6. 02	31
Ammonium sulfate	8. 9	490	57. 7	8. 46	20
DEAE-Sephadex	30. 0	461	10. 5	43. 9	19
Sephadex G–200	5. 0	225	0. 95	237	9

* Activity of H6PD in this fraction was very small, judging from a ratio of galactose 6-phosphate dehydrognease activity to glucose 6-phosphate dehydrogenase activity.

Crucian carp H6PD The microsomal fraction obtained in the above experiment was suspended in the buffer and sonicated for 10 min. using an Umeda sonicator. The sample was placed on two CM-Sephadex columns, No. 5, and the enzyme was eluted from each column by a linear gradient made with 300 ml portions of the buffer and of 50 mM Na_2HPO_4. Fractions of high H6PD activity were concentraed by ultrafiltration to 10 ml. The concentrated solution was treated with ammonium sulfate and the 25% (w/v) fraction was placed on a DEAE-Sephadex column, No. 1. The enzyme was eluted by a linear gradient made with 100 ml portions of the buffer with and without 1.0 M NaCl. Fractions of high H6PD activity were concentrated by a collodion bag and placed on a Sephadex G–200 column for gel filtration. The conditions were the same as described above. Fractions of high enzyme activity were concentrated by a collodion bag (specific activity, 6.83; Table 2 and Fig. 1).

Table 2. Purification of H6PD from crucian carp liver. Activity was assayed at pH 10.0 with G6P and NADP.

Step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)
Microsomal fraction	146	82. 6*	1, 220	0. 067	100
CM-Sephadex	100	31. 4	68. 4	0. 46	38
Ammonium sulfate	4. 2	11. 3	13. 9	0. 83	14
DEAE-Sephadex	30. 0	10. 2	6. 02	1. 69	12
Sephadex G–200	10. 0	6. 28	0. 92	6. 83	8

* This is calculated by: $y=0.88\ x$, where x is activity with Gal6P at pH 10.0; y, activity of H6PD with G6P and NADP at pH 10.0. The activity of G6PD on Gal6P+NADP at pH 10.0 is very small and neglected.

Rockfish G6PD Livers, 15 g, frozen at –70°C were homogenized in 5 volumes of the buffer and centrifuged at 10,000 ×g for 10 min. The supernatant was treated with ammonium sulfate and the 25%–30% (w/v) fraction was placed on a CM-Sephadex column, No. 2. The enzyme was eluted by a linear gradient made with 200 ml portions of the buffer and of 0.1 M Na_2HPO_4. Fractions of high G6PD activity were made 30% (w/v) in respect to ammonium sulfate and the precipitate was dialyzed against the buffer. The specific activity of this solution was 2.0.

Lamprey G6PD Livers, 10 g, stored at –70°C were homogenized in 5 volumes of the buffer and centrifuged at 10,000 × g for 10 min. The 20%–30% (w/v) ammonium sulfate fraction of the supernatant was placed on a DEAE-Sephadex column, No. 2, and the enzyme was eluted as described for crucian carp G6PD. Fractions of high G6PD activity were dialyzed and concentrated by ultrafiltration. The specific activity of this solution was 0.22.

Results and Conclusion

Properties of G6PD

The substrate specificity of G6PD was strikingly similar among the enzymes from toad, frog, crucian carp, rockfish and lamprey as shown in Table 3. The *Km* values for G6P, Gal6P and dG6P at pH 7.5 fell within a narrow range of 18–40 μM, 4.0–10.0 mM and 1.3–3.3 mM, respectively, the values being comparable with those reported with G6PD from various animals including ungulates (Smith and Holdridge 1967), rodents (Glock and McLean 1953, Matsuda and Yugari 1967, Kimura and Yamashita 1972, Hori and Sado 1974), man (Marks *et al.* 1961, Kirkman 1962, Yoshida 1966, 1967) and echinoderms (Mochizuki and Hori 1976).

The effect of pH on G6PD activity was also similar among the enzymes studied (Figs. 2–6) and was comparable with that on rat and echinoderm G6PD.

Mg ions had no or a slightly stimulative effect on fish and amphibian G6PD as on rat G6PD (Glock and McLean 1953, Hori and Sado 1974), while PCMB and DEA were inhibitory to the enzymes as to rat G6PD. It was noteworthy that frog G6PD and lamprey G6PD were less affected by PCMB and DEA, respectively (Table 4).

Properties of H6PD

The properties of H6PD from toad and crucian carp were similar in regard to the *Km*'s for the various substrates (Tables 5 and 6) and to the effect of pH on the activity (Figs. 7 and 8). The *Km*'s reported by several investigators with the enzymes from trout (Shatton *et al.* 1971, Stegeman and Goldberg 1972), mouse (Beutler and Morrison 1967), rat (Hori and Sado 1974) and starfish (Mochizuki and Hori 1976) were also comparable with those of crucian carp and toad, except that the *Km*'s for glucose in the presence of NAD was lower in fishes and amphibians

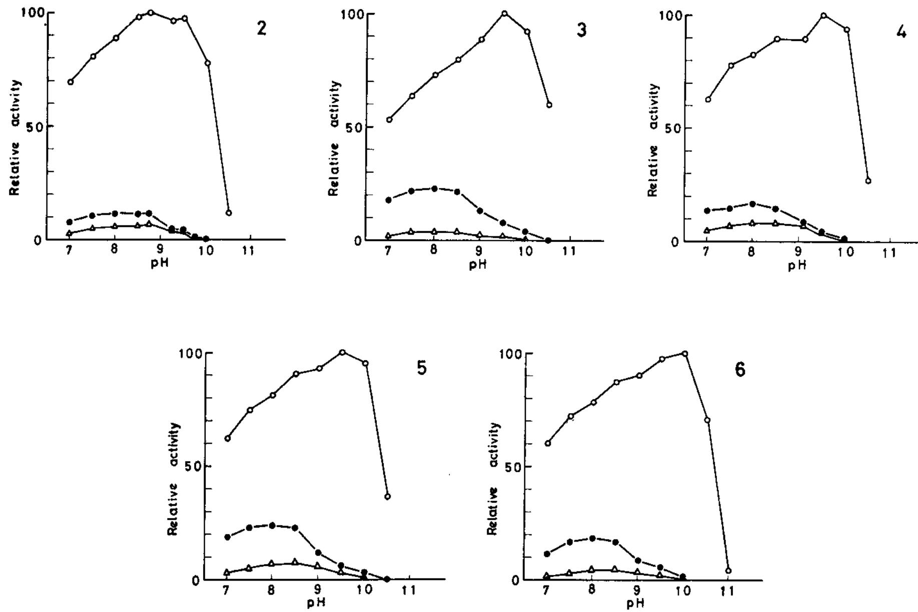
Table 3. Substrate specificity of G6PDs. Assays were performed with the partially purified enzyme at pH 7.5 and pH 10.0 at various substrate concentrations ranging above and below the *Km* values. The *Km* values were obtained from Lineweaver-Burk plots. *V* is given at percentage of activity with G6P and NADP at pH 10.0.

Species	Substrate	pH 7.5		pH 10.0	
		Km (mM)	*V* (%)	*Km* (mM)	*V* (%)
Toad	G6P	0.034	80	2.1	100
	Gal6P	10	26		
	dG6P	3.3	6		
	NADP (G6P)	0.006		0.045	
Frog	G6P	0.022	66	0.86	100
	Gal6P	6.7	33		
	dG6P	1.4	3		
	NADP (G6P)	0.009		0.059	
Crucian carp	G6P	0.025	54	2.3	100
	Gal6P	5.0	21		
	dG6P	2.0	6		
	NADP (G6P)	0.007		0.015	
Rockfish	G6P	0.040	65	0.71	100
	Gal6P	4.0	30		
	dG6P	1.3	5		
	NADP (G6P)	0.017		0.048	
Lamprey	G6P	0.018	74	0.56	100
	Gal6P	5.0	38		
	dG6P	1.9	5		
	NADP (G6P)	0.004		0.020	

No activity was detected on glucose with NADP, and also on NAD with the four substrates tested.

Table 4. Effect of chemical substances on enzyme activity. Assays were performed at pH 7.5 with G6P and NADP in the presence of chemicals. The values are presented as percentage of control (averages of triplicate determinations).

Enzyme	*p*-Chloromercuribenzoate (1 mM)	Dehydroepiandrosterone (70 μM)	$MgCl_2$ (10 mM)
Toad H6PD	195	100	64
Crucian carp H6PD	71	100	58
Toad G6PD	22	32	101
Frog G6PD	71	31	100
Crucian carp G6PD	20	37	122
Rockfish G6PD	9	34	113
Lamprey G6PD	10	71	107



Figs. 2–6. Effect of pH on G6PD of toad (Fig. 2), frog (Fig. 3), crucian carp (Fig. 4), rockfish (Fig. 5) and lamprey (Fig. 6). Substrate and NADP concentrations were 6.0 mM and 0.6 mM, respectively. Activity was expressed as percentage of the peak activity with G6P. ○——○, G6P; ●——●, Gal6P; △——△, dG6P.

Table 5. Substrate specificity of toad H6PD. Assays were performed with the partially purified enzyme at pH 7.5 and pH 10.0 at various substrate concentrations ranging above and below *Km* values. The *Km* values were obtained from Lineweaver-Burk plots. *V* is given as percentage of activity with G6P and NADP at pH 10.0.

Substrate	pH 7.5				pH 10.0			
	NADP		NAD		NADP		NAD	
	Km (mM)	*V* (%)	*Km* (mM)	*V* (%)	*Km* (mM)	*V* (%)	*Km* (mM)	*V* (%)
G6P	0.003	13	0.001	1	0.10	100	0.020	21
Gal6P	0.14	63	0.008	13	11	164	1.9	78
dG6P	1.3	45	0.065	33	56	51	11	30
Glucose	320	16	140	76	200	133	110	379
NADP (G6P)	0.002				0.007			
NAD (G6P)			0.001				0.002	

(0.03–0.2 M) as in pigs and cows (60–70 mM; Metzger *et al.* 1965) than in rodents and starfish (0.1–1.7 M; Beutler and Morrison 1967, Hori and Sado 1974, Mochizuki and Hori 1976).

Table 6. Substrate specificity of crucian carp H6PD. Assays were performed according to the method described in Table 5. V is given as percentage of activity with G6P and NADP at pH 10.0.

Substrate	pH 7.5				pH 10.0			
	NADP		NAD		NADP		NAD	
	Km (mM)	V (%)	Km (mM)	V (%)	Km (mM)	V (%)	Km (mM)	V (%)
G6P	0.002	16	0.001	3	0.14	100	0.036	19
Gal6P	0.009	27	0.003	6	0.78	128	0.18	33
dG6P	0.18	54	0.020	25	50	240	6.7	140
Glucose	310	47	100	125	290	165	74	275
NADP (G6P)	0.002				0.008			
NAD (G6P)			0.001				0.001	

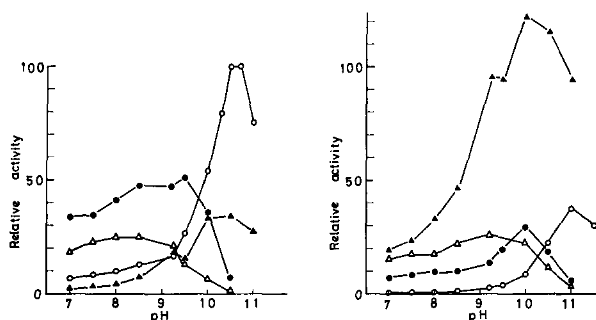


Fig. 7. Effect of pH on toad H6PD. Left, activity with NADP; right, activity with NAD. Concentration of substrate: G6P, Gal6P, dG6P, 6.0 mM; glucose, 0.2 M; NADP and NAD, 0.6 mM. Activity was expressed as percentage of the peak activity with G6P and NADP. ○——○, G6P; ●——●, Gal6P; △——△, dG6P; ▲——▲, glucose.

The pH dependence of H6PD activity was much more prominent than that of G6PD in crucian carp and toad as in trout and rat (Stegeman and Goldberg 1972, Hori and Sado 1974), and was quite different with substrates. The pH optima shifted to higher pH levels when NAD was replaced for NADP. The Km's for the four substrates were significantly lower in the presence of NAD than NADP. The similar characters have been reported with trout and rat H6PD (Shatton *et al.* 1971, Stegeman and Goldberg 1972, Hori and Sado 1974).

Mg ions had an inhibitory effect, but DEA had no effect on toad and crucian carp H6PD as on trout and rat H6PD (Shatton *et al.* 1971, Stegeman and Goldberg 1972, Hori and Sado 1974).

Crucian carp H6PD was less affected by PCMB than its G6PD as reported with rat H6PD and G6PD (Hori and Sado 1974), while toad H6PD was drastically

stimulated by PCMB. Stimulation by PCMB has not been reported with any other G6PD or H6PD (Table 4).

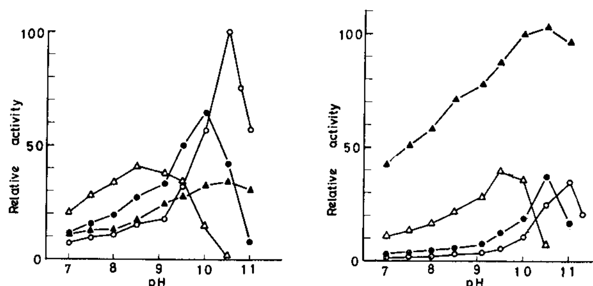


Fig. 8. Effect of pH on crucian carp H6PD. Left, activity with NADP; right, activity with NAD. Substrate and coenzyme concentrations were the same as in Fig. 7. Activity was expressed as percentage of the peak activity with G6P and NADP. ○——○, G6P; ●——●, Gal6P; △——△, dG6P; ▲——▲, glucose.

Molecular weights of crucian carp G6PD and H6PD

Molecular weights of G6PD and H6PD from crucian carp (Funa) were previously reported to be 28×10^4 and 23×10^4, respectively (Hori *et al.* 1975). Re-examination of the values by the method of Zwaan (1967) with the purified enzyme yielded the respective values of 25×10^4 and 23×10^4. Upon electrophoresis on SDS-polyacrylamide gel (Shapiro *et al.* 1967), each sample exhibited due to impurities some minor protein bands in addition to the major band which might probably be the enzyme polypeptides. From the calibration curve with standard proteins, the molecular weights of these polypeptides were estimated as 5.6×10^4 for G6PD and 9.9×10^4 for H6PD. This suggests that G6PD might exist as a tetramer, while H6PD exists as a dimer.

G6PDs from various species of fishes and amphibians move more slowly than G6PDs from reptiles, aves and mammals on polyacrylamide gels (Kamada and Hori 1970, Machizuki and Hori 1973) and the molecular weights of the former were about twice as much as those of the latter (Hori *et al.* 1975). Such a difference might be explained by either (1) the difference in size of polypeptides, or (2) the difference in the electric charges, or (3) the difference in the quarternary structure. The results of the present study seem to be favorable to the third explanation. This is in agreement with the conclusion of Yamauchi and Goldberg (1973) drawn from genetic analysis of some G6PD mutants in trout that the enzyme is tetrameric. It appears thus likely that the size of G6PD gene would not have undergone a drastic change during vertebrate evolution.

Summary

Glucose 6-phosphate dehydrogenase (G6PD) and hexose 6-phosphate dehydrogenase (H6PD) have been isolated from crucian carp, toad, frog, rockfish and lamprey, and their properties were studied with respect to *Km*'s for substrates and to effects of pH, *p*-chloromercuribenzoate, dehydroepiandrosterone and magnesium ions.

The results have indicated that G6PD and H6PD of fishes and amphibians possess strikingly similar catalytic properties to those of rat, respectively.

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