Further Studies on Animal Glucose 6-Phosphate Dehydrogenases

By

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(With 7 Tables)

The animal enzymes which are active on glucose 6-phosphate (G6P) in the presence of nicotinamide adenine dinucleotide phosphate (NADP) occur in two forms; one has narrow substrate and coenzyme specificities and the other had broad substrate and coenzyme specificities. The former which is active on G6P -NADP, but not on G6P-nicotinamide adenine dinucleotide (NAD) is commonly called glucose 6-phosphate dehydrogenase (G6PD), whereas the latter which is active on both G6P-NADP and G6P-NAD is designated by Ohno *et al.* (1966) as hexose 6-phosphate dehydrogenase (H6PD). Beutler and Morrison (1967) mentioned that H6PD was identical with the enzyme formerly known as glucose dehydrogenase (GD).

It has been known that G6PD (in a narrow sense) occurs widely in various invertebrate and vertebrate animals, while recent studies on H6PD revealed that H6PD was also common at least in vertebrates (Beutler and Morrison, 1967; Mandula *et al.* 1970; Metzger *et al.*, 1964, 1965; Ohno *et al.*, 1966; Shatton *et al.*, 1971; Shaw, 1966; Srivastava *et al.*, 1972; Stegeman and Goldberg, 1971). In addition, genetic studies by several workers (Mathai *et al.*, 1966; Ohno *et al.*, 1965; Porter *et al.*, 1962; Ruddle *et al.*, 1968; Shaw and Barto, 1965; Trujillo *et al.*, 1965; Young *et al.*, 1964) indicated that erythrocyte G6PDs of human, horse, donkey and hare, and *Drosophila* G6PD were sex-linked, while H6PDs of *Peromyscus* and mouse were autosome-linked.

Working on the enzymes from a variety of animals, Kamada and Hori (1970) have found that the animal G6PDs (in a broad sense) exist in various molecular forms which can be classified into three major types in regard to their substrate specificity; the first designated as type I G6PD is active on G6P alone, type II G6PD is as active on galactose 6-phosphate (Gal6P) as on G6P and type III G6PD is equally active on G6P, Gal6P and 2-deoxy-D-glucose 6-phosphate (dG6P). Type I G6PD is common in invertebrates and vertebrates and type II G6PD occurs in some invertebrates and in lower vertebrates, while type III G6PD is found only in vertebrates. According to this classification, H6PD belongs to type III G6PD.

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It was thus possible to speculate that type III G6PD (H6PD or GD) might have evolved from type I G6PD and that type II G6PD might be the intermediate form between type I and type III G6PDs or in other words, between G6PD (in a narrow sense) and H6PD or GD.

Hori and others also reported that type I G6PD, but not type III G6PD of rat liver and of human leucocytes was inducible by estradiol (Hori, 1968; Hori and Matsui, 1967; Matsui *et al.*, 1968). Consequently, it is feasible that not only the genes for G6PD and H6PD differ, but also the mechanisms involved in the expression of these genes differ from each other.

The present study was undertaken in order to ascertain whether type I G6PD would actually be an ancestral molecule of type III G6PD. For this purpose, the coenzyme specificity as well as the substrate specificity were examined with enzymes from 68 species belonging to ten phyla, by scanning the stained electrophoretic gels in a densitometer.

Materials and Methods

Enzyme preparation: The animals used in this study are listed in Table 1. Parts or whole bodies of the animals (mostly livers in vertebrates) were homogenized with one or two parts of 0.1% Triton X-100 in 20 mM Tris-maleate buffer, pH 7.2, containing 5 mM ethylenediaminetetraacetate (EDTA), and centrifuged at 15,000 $\times$g for 30 min. During the course of this study, it was found that G6PDs, especially type I enzymes, rapidly lost their activity by repeated freeze-thawing in the presence of Triton X-100. Therefore, in later experiments, extracts homogenized with the buffer containing Triton X-100 were dialyzed against the Triton-free buffer. Homogenization with Triton-free buffer was also carried out. The resultant supernatants and dialyzed enzyme solutions were used for electrophoresis. In the case of rat, partial purification was carried out in order to separate different molecular forms.

As previouly reported, rat G6PDs were composed of seven molecular forms, designated as A, B, C, D, E, E_1 and F in an increasing order of electrophoretic mobility (Hori *et al.*, 1966; Hori and Kamada, 1967). Band A, C, E and E_1 enzymes belong to type III G6PD, and band D and F enzymes belong to type I G6PD on the basis of their substrate specificity (Kamada and Hori, 1970). Band B enzyme exhibited nothing dehydrogenase activity, i.e., it was active in the absence of substrate (Hori *et al.*, 1967; Kamada and Hori 1970).

Rat livers were homogenized with one part of 0.1% Triton X-100 in 20 mM Tris-maleate buffer, pH 7.2, containing 5 mM EDTA, and sonicated. After dilution with the Triton-free buffer, the pH of homogenates was brought to 5 with concentrated acetic acid and the resultant precipitate was removed by centrifugation. Solid ammonium sulfate (25 g/100 ml) was added to this supernatant and the precipitate (AS25 ppt) was collected by centrifugation. Furthermore, ammonium sulfate (10 g/100 ml) was added to the AS25 supernatant and the precipitate (AS35 ppt) was collected by centrifugation. AS25 ppt contained mainly band C, E and E_1 enzymes, and a small amount of band F enzyme. In contrast, AS35 ppt contained mainly band F enzyme, and small amounts of band C, E and E_1 enzymes. Both precipitates were dissolved separately in a small volume of 20 mM phosphate buffer, pH 6.3, containing 5 mM EDTA, and dialyzed against the same buffer. The dialyzed AS25 solution was placed on DEAE-Sephadex A-50 column previously equilibrated with the same buffer, and eluted successively with the same buffer and 50 mM

Table 1. List of species examined

Species		Organs examined
PORIFERA		
Sponge	*Suberites* sp.	W
COELENTERATA		
Hydra	*Pelmatohydra robusta*	Wp
Sea anemone 1	*Anthopleura midori*	W
Sea anemone 2	*Anthopleura kurogane*	W
Sea anemone 3	*Tealia felina coriacea*	W
Sea anemone 4	*Metridium senile fimbriatum*	W
PLATYHELMINTHES		
Fresh-water planarian 1	*Polycelis auriculata*	Wp
Fresh-water planarian 2	*Polycelis sapporo*	Wp
Fresh-water planarian 3	*Dugesia japonica*	Wp
Land planarian	*Bipalium fuscolineatum*	Wp
Polyclad	*Notoplana humilis*	Wp
NEMERTINEA		
Ribbon worm	*Tubulanus punctatus*	W
MOLLUSCA		
GASTROPODA		
Neptune whelk	*Neptunea polycostata*	L
River snail	*Semisulcospira bensoni*	Wp
Land snail 1	*Ezohelix flexibilis*	L
Land snail 2	*Euhadra peliomphala*	L
Amber snail	*Succinea lauta*	Wp
BIVALVIA		
Scallop	*Patinopecten yessoensis*	L, Gi
Oyster	*Crassostrea gigas*	L
Brackish clam	*Corbicula japonica*	Wp
Drain clam	*Sphaerium lucustre japonicum*	Wp
Japanese littleneck	*Tapes japonica*	Wp
Clam	*Meretrix lusoria*	L
Trough shell	*Spisula sachalinensis*	L
CEPHALOPODA		
Squid	*Ommastrephes sloani pacificus*	L, Gi
ANNELIDA		
Tubeworm	*Pseudopotamilla occelata*	Wp
Earthworm	*Tubifex hattai*	Wp
Leech	*Barbronia weberi*	Wp
ARTHROPODA		
Crayfish	*Procambarus clarki*	L, Gg
Cockroach	*Blattella germanica*	Wp
Asiatic locust	*Locusta migratoria*	W
False rice grasshopper	*Parapleurus alliaceus*	Wp
Katydid	*Metrioptera japonica*	Wp
Ground beetle	*Anisodactylus signatus*	W
Fruit fly	*Drosophila melanogaster*	Wp
ECHINODERMATA		
Sea cucumber	*Stichopus japonicus*	Gu
Sea star 1	*Asterina pectinifera*	Gu, Go
Sea star 2	*Asterias amurensis*	Gu, Go
Sea star 3	*Aphelasterias japonica*	Gu, Go

Table 1. (Continued)

Species		Organs examined
Sea urchin 1	*Strongylocentrotus intermedius*	Gu, Go
Sea urchin 2	*Strongylocentrotus nudus*	Gu, Go
PROTOCHORDATA		
Sea squirt 1	*Halocynthia roretzi*	Gu
Sea squirt 2	*Halocynthia aurantium*	Gu
VERTEBRATA		
AGNATHA		
Lamprey	*Entosphenus japonicus*	L
OSTEICHTHYES		
Clupeida		
Rainbow trout	*Salmo gairdnerii irideus*	L
Sockeye salmon	*Oncorhynchus nerka adonis*	L
Cyprinida		
Crucian	*Carassius carassius*	L
Bitterling	*Acheilognathus* sp.	L
Dace	*Tribolodon hakonensis*	L
Loach 1	*Barbatula toni*	L
Loach 2	*Lefua nikkonis*	L
Loach 3	*Misgurnus anguillicaudatus*	L
Anguillida		
Eel	*Anguilla japonica*	L
Cyprinodontida		
Medaka	*Oryzias latipes*	Wp
Gasterosteida		
Stickleback	*Pungitius sinensis*	L, Gu
Cottida		
Sculpin	*Cottus* sp.	L
Percida		
Sandfish	*Arctoscopus japonicus*	L
AMPHIBIA		
Salamander	*Hynobius retardatus*	L
Newt	*Cynopus pyrrhogaster pyrrhogaster*	L
Clawed toad	*Xenopus laevis*	L
Tree frog	*Hyla arborea japonica*	L
Frog 1	*Rana chensinensis*	L
Frog 2	*Rana nigromaculata*	L
REPTILIA		
Red-eared turtle	*Psedemys scripta elegans*	L
Snake	*Elaphe climacophora*	L
MAMMALIA		
Rat	*Rattus norvegicus*	L
Mouse	*Mus musculus*	L
Dog	*Canis familairis*	L

Abbreviations: W, a single whole body; Wp, many whole bodies; L, liver; Gg, green gland; Gi, gill; Go, gonad; Gu, gut.

NaCl in the buffer. The effluent with 50 mM NaCl was treated with ammonium sulfate (25 g/100 ml) and the precipitate was collected. On the other hand, the dialyzed AS35 solution was placed on the same column and washed with 100 mM NaCl in the same buffer, and eluted with 500 mM NaCl in the buffer. The 500 mM NaCl effluent was treated with ammonium sulfate (35 g/100 ml) and the resultant precipitate was collected. The precipitates were dissolved separately in a small volume of the same buffer and dialyzed against the buffer. The dialzyed enzyme solutions were used for electrophoresis. The 50 mM NaCl fraction from AS25 ppt contained band C, E and E_1 enzymes, while the 500 mM fraction from AS35 ppt contained band F enzyme. The partial purification of crucian G6PDs was carried out by the extraction from acrylamide gel after electrophoresis.

Polyacrylamide gel electrophoresis: Electrophoresis was carried out by the method of Ornstein (1964) and Davis (1964), employing 7.5% acrylamide in the running gel and 3.3% acrylamide in the sample gel. The gel was cast into $3\times12\times80$ mm columns for qualitative observations or $1\times60\times70$ mm columns for densitometry.

Substrate and coenzyme specificities: After electrophoresis, the gel was vertically cut into eight to ten strips 5 mm width and stained for 1 hour at 37°C in the incubating medium containing G6P or Ga16P or dG6P, 2.2 mM, or glucose, 0.1 M; NADP or NAD, 0.2 mM; nitro blue tetrazolium, 0.3 mM; phenazine methosulfate, 0.07 mM; KCN, 10 mM; $MgCl_2$, 10 mM; Tris-maleate buffer, pH 7.2, 50 mM. The stained gels were then photographed and scanned in a densitometer. The enzyme activity was expressed as the integrated area of a densitometric scanning, and the relative enzyme activities on various substrates and coenzymes were expressed as percentages of the activity on G6P-NADP.

Heat stability: After electrophoresis, the gel ($1\times60\times70$ mm) was vertically cut into six strips of 5 mm width. They were divided into two groups consisting of every other strips; one was placed in a refrigerator until staining, while the other was heated at 50°C for 5 min in a Petri dish containing pieces of wet filter papers. Both groups were then incubated at 37°C for 1 hour in the staining medium using G6P as substrate and NADP as coenzyme. The stained gel strips were photographed and scanned in a densitometer. The enzyme activity of heated samples expressed as percentage of the untreated control was taken as a measure of the heat stability.

Results

The results on the substrate and coenzyme specificities and the heat stability are given in Tables 2–6. Animal G6PDs can be classified into two major types in regard to their coenzyme specificity; one is able to utilize both NADP and NAD as coenzyme and the other NADP alone. They are tentatively designates as N,N-G6PD and N-G6PD, respectively.

N-G6PD

All N–G6PDs were inactive on glucose, but their relative activities on Ga16P and dG6P varied in different enzymes, ranging from 0 to 42% of the activity on G6P. Among 34 enzymes examined, 21 had the activity on Ga16P ranging from 0 to 9.7%, 10 had the activity ranging from 10 to 16.6% and 3 had the activity more than 36%. The enzymes of hydra and squid belonged to the 3rd group.

The activity on dG6P was less than 5% of that on G6P in most enzymes (30 out of 34) and 7.5 to 12.8% in 4 enzymes. The enzymes of hydra and squid were inactive or only slightly active on dG6P. Accordingly, it is clear that the

Table 2. Substrate specificity fo N-G6PD

Species	G6P	Gal6P	dG6P	Glucose
PORIFERA				
Sponge	100	15.5±0.2(2)	12.8±0.2(2)	0(2)
COELENTERATA				
Hydra Band A	100	36.4±3.4(4)	1.7±0.4(3)	0(4)
Band B	100	36.4±2.5(4)	0 (4)	0(4)
PLATYHELMINTHES				
Land planarian	100	6.2±0.2(2)	1.3±0.1(2)	0(2)
Fresh-water planarian 1	100	4.2±0.2(2)	1.6±0.1(2)	0(2)
Fresh-water planarian 2	100	4.4±0.2(3)	1.4±0.3(2)	0(3)
NEMERTINEA				
Ribbon worm	100	6.7±0.3(4)	1.0±0 (4)	0(4)
MOLLUSCA				
Neptune whelk	100	16.6±0.3(8)	7.5±0.8(7)	0(8)
River snail	100	15.3±0.8(2)	12.4±0.6(2)	0(2)
Land snail 1	100	4.6±0.6(2)	3.1±0.2(2)	0(2)
Land snail 2	100	8.6±0.2(2)	3.0±0.2(2)	0(2)
Oyster	100	1.9±0 (2)	1.0±0.1(2)	0(2)
Squid	100	42.0±0.5(4)	0 (4)	0(4)
ANNELIDA				
Earthworm	100	1.7±0.1(4)	0.3±0 (4)	0(4)
Leech	100	3.6±0.3(4)	2.4±0 (4)	0(4)
ARTHROPODA				
Crayfish	100	9.7±0.2(3)	5.3±0.1(3)	0(3)
False rice grasshopper	100	7.4±0.1(2)	5.1±0.2(2)	0(2)
ECHINODERMATA				
Sea urchin 2	100	4.4±0.5(2)	5.1±0.8(2)	0(2)
VERTEBRATA				
PISCES				
Lamprey Band A	100	6.3 (1)	4.3±0.6(2)	0(3)
Band B	100	11.1 (1)	0 (1)	0(1)
Rainbow trout	100	5.6±1.0(2)	2.7±0.7(2)	0(2)
Loach 1	100	13.4±1.1(3)	2.6±0.4(2)	0(3)
Loach 2	100	5.7±0.4(2)	0 (2)	0(2)
Eel	100	15.1±0.8(3)	3.2±0.6(2)	0(3)
AMPHIBIA				
Newt	100	5.8 (1)	3.5±0.1(2)	0(1)
Salamander	100	0 (2)	0 (4)	0(2)
Clawed toad	100	0 (2)	0 (4)	0(2)
Tree frog	100	7.5 (1)	2.1 (1)	0(2)
Frog 1	100	12.2±1.0(4)	2.8±0.4(2)	0(4)
Frog 2	100	10.0 (1)	0.5 (1)	0(1)
REPTILIA				
Red-eared turtle	100	0 (2)	0 (2)	0(2)
Snake	100	8.1 (1)	3.5 (1)	0(2)
MAMMALIA				
Rat Band F	100	11.0±0.2(8)	12.4±0.8(7)	0(8)
Mouse	100	11.3 (1)	4.3 (1)	0(5)

Values given are relative to the activity on G6P-NADP. Values are averages ± the standard error with the number of determinations in parentheses.

Table 3. Substrate

Species	NADP		
	G6P	Gal6P	dG6P
MOLLUSCA			
Scallop L	100	21.3± 1.0(2)	0 (2)
Gi	100	32.3± 0.8(2)	0 (2)
ECHINODRMATA			
Sea cucumber Band A	100	68.2± 0.9(2)	9.5± 0.7 (3)
Band B	100	10.0± 1.5(2)	0 (2)
Sea urchin 2	100	10.2± 0.7(2)	4.4 (1)
Sea star 1	100	88.8 (1)	213.8 (1)
Sea star 2	100	255.9± 6.8(4)	52.4± 5.0 (3)
VERTEBRATA			
Pisces			
Rainbow trout	100	101.4± 5.8(4)	29.0± 1.6 (3)
Crucian	100	79.7± 5.2(3)	35.0± 3.2 (2)
Loach 1	100	63.7± 5.4(2)	8.9± 0.3 (2)
Loach 2	100	86.9± 4.6(2)	11.1± 0.6 (2)
Eel	100	114.8± 7.7(2)	141.6± 2.7 (3)
Medaka	100	76.4± 6.0(4)	21.0± 2.0 (4)
Stickleback	100	86.4± 3.5(3)	15.5± 0.1 (2)
Sculpin	100	96.5 (1)	12.4 (1)
Amphibia			
Salamander	100	67.7± 0 (2)	8.5± 0.4 (4)
Clawed toad Band B	100	79.1± 3.3(2)	19.8± 0.9 (4)
Band C	100	128.4± 2.5(2)	15.1± 0.3 (4)
Tree frog	100	44.6± 2.5(2)	9.7± 0.6 (2)
Frog 1 Band C	100	41.3± 3.5(4)	13.6± 1.8 (2)
Band D	100	134.3±20.3(4)	22.4± 1.8 (3)
Reptilia			
Red-eared turtle	100	178.0±18.7(2)	329.5± 7.3 (2)
Mammalia			
Rat Band C	100	81.5± 3.0(9)	113.3± 4.3(13)
Mouse	100	109.8± 9.3(4)	218.4±24.6 (4)

Values given are relative to the activity on G6P-NADP. Values are averages

enzyme structure favorable to the activity on Gal6P would not be necessarily so for the enzyme to act on dG6P. However, the reverse was not true; the enzymes active on dG6P were all active on Gal6P as well.

The enzymes from sponge, neptune whelk and river snail were peculiar among other invertebrate enzymes in that they were fairly active on both Gal6P and dG6P.

As is clear in the above, the majority of N-G6PDs were relatively inactive on Gal6P and dG6P. This is in good agreement with the data on G6PDs of human erythrocytes, leucocytes and liver, and *Neurospora* G6PD by Chan *et al.* (1972), Kirkman (1962), Messina *et al.* (1972), Ohno *et al.* (1966), Scott and Tatum (1971) and Yoshida *et al.* (1971), indicating that the activity on Gal6P is 8 to 14%, and that on dG6P is less than 4 % of that on G6P.

specificity of N,N-G6PD

	NAD			
Glucose	G6P	Gal6P	dG6P	Glucose
0 (2)	26.4±0.6(2)	21.3±1.7(2)	0 (2)	0 (2)
0 (2)	49.5±4.4(2)	20.5±0.8(2)	0 (2)	0 (2)
0 (3)	21.2±1.2(2)	37.9±5.2(2)	3.5± 0.1(2)	0 (3)
0 (3)	26.0±1.0(2)	4.2±0.1(2)	0 (3)	0 (3)
0 (2)	5.1±0.5(2)	4.0±1.0(2)	1.7± 0.3(2)	0 (2)
0 (3)	63.8 (1)	58.8 (1)	193.8 (1)	10.0 (1)
6.9±0.6(4)	47.7±4.4(4)	206.1±8.3(3)	50.5± 5.8(3)	8.2± 0.1(2)
33.0±0.9(3)	59.4±4.7(4)	82.2±4.6(4)	50.3± 9.2(4)	77.0± 5.8(4)
19.8±1.6(2)	43.8±5.7(3)	67.5±0.8(2)	31.2± 4.5(3)	39.1± 4.3(2)
50.0±3.6(3)	30.2±2.0(3)	44.9±1.2(3)	17.6± 0.8(2)	68.8± 0 (2)
43.1±2.3(2)	38.7±2.1(2)	61.4±3.2(2)	25.0± 1.3(2)	67.5± 3.5(2)
45.8±1.9(2)	41.3±1.9(3)	63.5±3.3(2)	141.6±19.7(3)	133.0±19.4(3)
19.6±0.4(4)	16.5±1.6(3)	25.1±3.8(4)	33.1± 5.6(4)	39.7± 6.9(4)
2.4±0.4(3)	56.8±1.1(2)	64.7±2.8(2)	24.8± 1.4(3)	17.1± 1.3(2)
2.3 (1)	28.3 (1)	36.1 (1)	18.9 (1)	12.0 (1)
0 (2)	24.6±0 (2)	33.8±0 (2)	29.2± 0 (2)	15.4± 0 (2)
8.5±0.4(2)	16.9±0.7(2)	28.2±1.2(2)	14.1± 0.6(2)	76.2± 3.2(2)
22.7±0.5(2)	26.4±0.5(2)	56.7±1.1(2)	3.8± 0.1(2)	60.4± 1.1(2)
1.5±0.1(2)	10.2 (1)	14.6±0.2(2)	30.2± 0.2(2)	10.5 (1)
10.1 (1)	9.4±0.8(3)	22.6±4.5(4)	15.2± 0.5(3)	10.2± 3.5(2)
6.2±1.2(2)	17.1±3.4(4)	70.5±2.4(3)	47.2± 1.2(2)	16.5± 1.8(2)
45.4±4.7(2)	45.6±1.2(2)	105.0±5.0(2)	363.0±37.0(2)	87.6± 2.4(2)
20.0±1.7(9)	66.4±2.4(9)	50.8±1.8(9)	129.2± 8.7(9)	39.9± 2.8(9)
20.8±4.9(4)	52.8±8.5(4)	60.9±4.2(2)	178.7±13.5(4)	43.5± 8.2(4)

± the standard error with the number of determinations in parentheses.

The heat stability index was less than 7 in 9 enzymes out of the 16 enzymes examined, and more than 11 in the rest. The highest value, 39.8 was obtained with land snail.

To be pointed out was that among the 5 vertebrate enzymes tested only the enzymes from loach was heat-labile, while rat and frog enzymes were comparatively heat-stable, and that the enzymes from neptune whelk and land snail were similarly heat-stable.

There was no correlation between the substrate and coenzyme specificities and the heat stability; for instance, the squid enzyme had a high Ga16PD activity, but was heat-labile, while the land snail enzyme had a narrow substrate specificity, but was heat-stable.

Table 4. Distribution of N- and N,N-G6PDs in Animal Kingdom

	Number of species examined	N-G6PD	N,N-G6PD
Porifera	1	+	−
Coelenterata	5	+	−
Platyhelminthes	5	+	−
Nemertinea	1	+	−
Mollusca			
Gastropoda	5	+	−
Bivalvia	6	+	−
(Scallop)	1	+	+
Cephalopoda	1	+	−
Annelida	3	+[a]	−
Arthropoda	7	+	−
Echinodermata			
Echinoidea	2	+	+
Holothuroidea	1	−	+
Asteroidea	3	−	+
Protochordata	2	*	*
Vertebrata			
Pisces	14	+	+[b]
Amphibia	6	+	+
Reptilia	2	+	+
Mammalia	3	+	+

+, −, N- or N,N-G6PD was present (+) or absent (−).
*, enzyme activity was not detected.
a, enzyme activity was not detected in *Pseudopotamilla occelata*.
b, N,N-G6PD activity was not detected in *Entosphenus japonicus*.

Table 5. Heat stability of N-G6PD

Species	Remaining activity (%)	Species	Remaining activity (%)
Hydra Band A	0 (9)	Crayfish Band A	0 (9)
Band B	11.1±0.7(9)	Band B	0 (9)
Land planarian	0 (9)	Sea urchin 1	0 (9)
Neptune whelk	21.8±1.2(6)	Loach 1	0 (9)
Land snail 1	39.8±2.7(6)	Salamander	11.7±1.4(7)
Squid	4.5±0.2(9)	Tree frog	20.8±0.8(6)
Earthworm	7.3±0.4(9)	Frog 1	29.4±0.5(6)
Leech	0 (9)	Rat	29.0±1.9(9)

Values given are relative to the activity of untreated control. Values are averages ± the standard error with the number of determinations in parentheses.

N,N-G6PD

The substrate specificity of the enzymes belonging to this type varied strikingly in different animals.

Table 6. Heat stability of N,N-G6PD

Species	Remaining activity (%)	Species	Remaining activity (%)
Scallop	0 (9)	Salamander	10.9±0.9(9)
Sea cucumber Band A	0 (9)	Tree frog	3.8±0.1(6)
Band B	0 (9)	Frog 1 Band C	0 (9)
Sea urchin 1	0 (9)	Band D	0 (9)
Sea star 2	0 (9)	Red-eared turtle	71.1±2.0(9)
Rainbow trout	86.4±1.7(6)	Rat Band C	92.2±2.3(7)
Loach 1	68.3±0.9(9)	Band E	87.3±2.4(8)
Stickleback	62.2±0.9(6)		

Values given are relative to the activity of untreated control. Values are averages ± the standard error with the number of determinations in parentheses.

The N,N-G6PD can be further divided into three group; the first was characterized by the low activity on Gal6P and dG6P. The enzymes from a scallop, *Patinopecten yessoensis*, a sea cucumber, *Stichopus japonicus*, and sea urchins, *Strongylocentrorus nudus* and *S. intermedius*, belonged to this group.

The second was as active on Gal6P as on G6P, but was less active on dG6P than on G6P. The enzymes from sea stars of order Forcipulata, most fishes and amphibians belonged to this group. Among these enzymes, those from two species of order Forcipulata, *Asterias amurensis*, and *Aphelasterias japonica*, were outstanding in that the activity on Gal6P was more than twice as high as that on G6P.

The third group including the enzymes from a sea star of order Spinulosa (*Asterina pectinifera*), eel, turtle, snake, rat, mouse and dog was characterized by its high activity on dG6P.

The heat stability of N,N-G6PD also varied in different animals; the heat stability index was zero in 5 invertebrate enzymes, and was more than 60 in fishes, turtle and rat, while those of amphibians were zero or very low.

As in the case of N–G6PD, there was here again no correlation between the substrate specificity and heat stability.

Discussion

In the present study, we surveyed the enzymes from 68 species belonging to ten phyla, including 32 species which were not examined by Kamada and Hori (1970).

Kamada and Hori classified animal G6PDs into types I, II and III on the basis of their substrate specificity using NADP as coenzyme. Their observation on the substrate specificity was however made by simply inspecting the stained electrophoretic gels, so that the results do not seem to be quantitative and accurate. On the other hand, the substrate specificity was quantitated in the present study by scanning the stained gels in a densitometer using both NAD and NADP as

coenzyme. Therefore, present results appear to be more quantitative than those by Kamada and Hori (1970), though the study with purified enzymes is required for more accurate investigations.

When comparing the classification proposed by Kamada and Hori (1970) with the present one, the following conclusion may be possible: 1) All the type I G6PDs are the N-G6PDs, while all the type III G6PDs are the N,N-G6PDs 2) The type II G6PDs of invertebrate animals (hydra, neptune whelk) belong to the N-G6PDs, while the type II G6PDs of vertebrate animals belong to the N,N-G6PDs 3) The N,N-G6PDs of a sea cucumber, *Stichopus japonicus*, sea urchins, *Strongylocentrotus intermedius* and *S. nudus*, and a scallop, *Patinopecten yessoensis*, which were not formerly investigated can be called the type II G6PD in the sense that they were the enzymes in a border line between the N-G6PD and the N,N-G6PD. It should be postponed, therefore, to determine which designations, types I to III or N-, N,N- are appropriate until quantitative studies of the substrate and coenzyme specificities are made with purified enzymes.

In any case, the present findings lend support to the hypothesis put forward by Kamada and Hori that type III G6PD might have evolved from type I G6PD.

N,N-G6PDs of vertebrate animals

As shown in Table 7, N,N-G6PDs of vertebrates can be divided into two distinct types based on the difference in activity on dG6P; one has a higher, and the other has a lower activity on dG6P than on G6P. The former is found in mammals and reptiles, and the latter in amphibians. Fishes have both types of enzymes. In addition, vertebrates also include two distinct types of N,N-G6PDs in respect to electrophoretic mobility; one is slower and the other is faster than N-

Table 7. Comparison of properties of G6PDs of various animals

	Electrophoretic mobility[1]	Heat stability	Activity on dG6P[2]	Activity on glucose[3]
N,N-G6PDs				
Mammalia	slow	stable	high	+
Reptilia	slow	stable	high	+
Amphibia	fast	labile	low	+
Pisces	slow or fast	stable	high or low	+
Echinodermata	slow	labile	high or low	+ or −
Mollusca		labile	none	−
N-G6PDs		slightly stable or labile	low or none	−

1) "Slow" (or "fast") indicates that N,N-G6PD is slower (or faster) than N-G6PD in a species containing both types of enzymes.
2) "High" or "low" indicates that the relative activity is more or less than 100, respectively. "None" indicates no activity.
3) Enzyme is active (+) or inactive (−) on glucose.

G6PD. The former is found in mammals and reptiles, and the latter in amphibians. Here again, fishes have both types of enzymes. This suggests the possibility that primitive fishes already contained a variety of N,N-G6PDs, some of which later on became ancestral molecules of the present-day amphibian or reptilian enzymes.

Heterogeneity of echinoderm enzymes

The present findings on the N,N-G6PDs of echinoderms are of particular interest; the enzymes of two species of sea urchins, *Strongylocentrotus nudus* and *S. intermedius*, and one species of sea cucumber, *Stichopus japonicus*, had very low activities on the substrates other than G6P in the presence of either NADP or NAD. In this respect, these enzymes were very much alikened to the N-G6PD. On the other hand, the enzymes of two species of order Forcipulata, *Asterias amurensis*, and *Aphelasterias japonica*, showed very high activities on Gal6P-NAD and Gal6P-NADP, while the enzyme of *Asterina pectinifera* (order Spinulosa) was highly active on dG6P-NAD and dG6P-NADP. In this respect, it can be said that two Forcipulata enzymes are alikened to the enzymes of fishes and amphibians, while the *Asterina* enzyme is alikened to the enzymes of turtle and mouse. Such variation of the enzyme characteristics in echinoderm animals which has not been found in other phyla strongly suggests the possibility that the gene coding for G6PD molecule suffered from frequent mutations during the evolution of echinoderm animals.

N,N-G6PD of scallop

It is also interesting that the N,N-G6PD has been found in a scallop, *Patinopecten yessoensis*. This enzyme has a similar characteristics to that of *Strongylocentrotus* in its rather narrow substrate specificicty. However, the N,N-G6PD has not been found in coelenterates and other protostomians; i.e., platyhelminthes, nemertinean, annelids, arthropods and other molluscs.

Physiological role of the N,N-G6PD or H6PD

The physiological meaning of GD activity of H6PD has been questioned because of its extremely high Km for glucose (Beutler and Morrison, 1967; Brink, 1953; Metzger *et al.*, 1964; Stegeman and Goldberg, 1971; Strecker and Korkes, 1952; Thompson and Carper, 1970), although the high Km value itself does not necessarily mean the physiological inertness, as evidenced by glucokinase (Schimke and Grossbard, 1968). In contrast, the Kms for Gal6P and dG6P of H6PD are extremely low, being well within a physiological range (Beutler and Morrison, 1967; Shatton *et al.*, 1961; Stegeman and Goldberg, 1971). However, these phosphorylated sugars have not been reported to occur in animals. Consequently, the Gal6PD or dG6PD activity of H6PD would not seem to be of any physiological meaning.

In relation to the physiological significance of rat liver H6PD, Srivastava and Beutler (1969) stated that this enzyme may serve physiologically as a G6PD rather than as a Gal6PD, possibly as source of NADPH in microsomal oxidase systems. They also reported that the reaction products of G6P and Gal6P by H6PD was 6-phosphogluconic acid and 6-phosphogalactonic acid, respectively, and that 6-phosphogluconate dehydrogenase could not utilize 6-phosphogalactonic acid in the presence of NADP. It would be thus less likely that all the pentose phosphate shunt enzymes exist in dual forms, one for G6P and its metabolites and the other for Gal6P and its metabolites.

In this regard, we would like to suggest a hypothesis that the physiological significance of the H6PD is in its ability of utilizing both NAD and NADP in the oxidation of G6P. It is well known that reduced NAD is primarily used as a potential source of energy-rich phosphate in the form of ATP, while reduced NADP has a primary role as a reducing agent. If this holds, the acquisition of NAD-utilizing ability of the G6PD molecule in higher animals seems to be purposive in view of their high energy requirement.

Summary

The glucose 6-phosphate dehydrogenases (G6PDs) from a variety of animals were compared using such parameters as the electrophoretic patterns, substrate and coenzyme specificities and heat stability. Four substrates, i.e., glucose 6-phosphate, galactose 6-phosphate (Gal6P), 2-deoxy-D-glucose 6-phosphate (dG6P) and glucose, and two coenzymes, i.e., NADP and NAD, were tested. As a result, the animal G6PDs were classified into two major types, NADP-dependent G6PDs (N-G6PDs) and NADP- and NAD-dependent G6PDs (N,N-G6PDs) based on the coenzyme specificity. The N-G6PD was specific to NADP, utilized Gal6P and dG6P at a small rate and was not active on glucose. The enzyme was found in a wide variety of vertebrates and invertebrates. The N,N-G6PD was found in vertebrates and higher invertebrates (echinoderms and a mollusc), and utilized both NADP and NAD as coenzyme. The vertebrate enzymes utilized all four substrates, while the echinoderm enzymes were slightly active or not active on glucose. On the other hand, a molluscan enzyme was not active on glucose and dG6P. The results suggested the possibility that the N,N-G6PD might have diverged from the N-G6PD during or prior to the evolution of echinoderms.

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